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Identification and Characterization of *Salmonella* Serotypes Isolated from Pork and Poultry from Commercial Sources

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IDENTIFICATION AND CHARACTERIZATION

OF *SALMONELLA* SEROTYPES

ISOLATED FROM PORK AND POULTRY FROM COMMERCIAL SOURCES

by

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Salmonella is one of the leading causes of foodborne illness worldwide, and it is estimated that 1.4 million infections occur annually in the U.S. alone. The Premi®Test *Salmonella* (PTS), is a potential tool for rapid detection and identification of *Salmonella* serovars. The objective of this project was to evaluate the use of the PTS system as a serotyping tool to identify pork and poultry isolates obtained from vertically integrated operations and to characterize their antibiotic resistance. In addition a risk assessment model was proposed for future research.

Two hundred isolates were evaluated. All isolates were serotyped using the traditional Kaufmann-White scheme and the PTS system. Among the isolates 63 different serotypes were represented, 36 of which were included in the PTS database and 27 were not present in it. CDC pulsed field gel electrophoresis protocol was used to characterize the relatedness among isolates and their antibiotic resistance was determined using the Kirby-Bauer disc diffusion test.

Serotype identification using the PTS system was reproducible independently of the source (pork or chicken) or replication. Sixty three percent of the serotypes present in by the PTS database were successfully indentified as *Salmonella* and matched traditional serotyping. Thirty seven percent of the isolates were identified as *Salmonella* but did not match results from traditional serotyping. Close relatedness among isolates was not responsible (in most of the cases) for the mismatches between KW and PTS system from serotypes present in the data base. Tetracycline resistance was observed mainly in pork isolates (*S*. Anatum, *S.* Heidelberg, *S*.Mbandaka and *S.* Johannesburg). Two multidrug resistance patterns were detected in *S*. Typhimurium and *S.* Bovis –morbificans (G-AM-C and Te-G-AM respectively).

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CHAPTER I

LITERATURE REVIEW

I. *Salmonella*

A. Introduction

Salmonella is cited as in the most common causative agent of foodborne illness (17), The United States alone reported an estimated 1.4 million total cases of non-thyphoidal *Salmonella* per year (31,64,77).The large number of outbreaks in developed and developing countries produced by this bacteria indicates its importance and impact (6). Salmonellosis is not only responsible of a large number of illnesses but also there is a cost associated with these outbreaks which in United States has been estimated to range from $600 million to $3.5 billion each year (78).

According to the preliminary report from FoodNet on the incidence of infection with foodborne pathogens, the ongoing efforts to reduce cases of salmonellosis associated with the consumption of contaminated meat, poultry, produce and other foods are showing success (21). This report indicates that fewer cultures of raw broiler chicken samples yielded *Salmonella* in 2009 (7.2%) than in 2006 (11.4%) (8). The United State Department of Agriculture (USDA) and the Food Safety and Inspection Service (FSIS) have established three categories for meat facilities according to the level of interventions that they have implemented toward the reduction of pathogens. On 2009, the percentage of broiler chicken slaughter establishments meeting FSIS's rigorous category 1 *Salmonella* contamination criteria increased from 49% in 2006 to 82% in 2009 (21).

Preventing *Salmonella* from contaminating food during the farm to table process remains challenging. The food industry, especially poultry and pork processors, are

highly regulated by government monitoring programs (52). Pre-requisite programs and HACCP plans are successful strategies already adopted by the meat industry. Interventions, which demonstrate effective reductions in the occurrence and levels of pathogenic bacteria at different processing steps, have been included in production lines to keep critical points under control. For example combinations of temperature and pressure on sprays with or without bactericides at different levels, steam with or without vacuum, irradiation, pulsed electric fields, high pressure, ultraviolet light and microwaves are some of the decontamination treatments being used for decontamination of pork and poultry carcasses (4, 6). Some of the chemical treatments include dioxide, acidified sodium chlorite, ozone, organic acids, trisodium phosphate (TSP) and cetylpyridinium chloride (CPC) (6, 45).

Surveillance and inspection programs have played a major role in the reduction of cases of food borne illnesses. Most developed countries have systems established to report the occurrence of outbreaks (55). Reports obtained from these surveillances are subjected to some limitations; 1) people do not always look for medical aid when they get infected, 2) physicians do not always request a stool culture of suspected cases; 3) not all positive cases are reported and shared in the database, and 4) differences in health-care seeking behaviors among age groups is variable. All these factors affect the accurate estimation of the amount of cases of illness caused by *Salmonella* (21, 55).

The World Health Organization (WHO) and The Food and Agriculture Organization (FAO) are two international networks addressing the problem of emerging *Salmonella* infections. The objective is to integrate countries and regions to provide training, information sharing, analysis of trends and to allow the immediate response in the

occurrence of outbreaks (55) and to achieve long-term sustainable results in food safety and quality around the world (29). The United States has an integrated program called FoodNet, that is a collaborative program among CDC, 10 state health departments, the USDA-FSIS, and the Food and Drug Administration (FDA) this program has conducted active, population-based surveillance for laboratory-confirmed cases of infection caused by *Campylobacter*, *Listeria*, *Salmonella*, STEC O157, *Shigella*, *Vibrio*, and *Yersinia* since 1996 (21).

Serotyping is an important tool to understand the epidemiology of *Salmonella* infections, and is frequently used to trace back sources of contamination during an outbreak .The serotyping scheme developed by White and Kauffmann on 1920 was based on the discovery of flagella H antigen, the somatic O antigen and the phase-shift in the H antigen (55) .The Kauffmann-White method, used worldwide, is consider the gold standard for identification of *Salmonella* serotypes. Identification of *Salmonella* serotypes provides information about the severity of the disease, the source of contamination and the resistance pattern (55).

Some *Salmonella* species are host adapted. *S*. Typhi and *S*. Paratyphi cause typhoid and paratyphoid fever in humans, *S*. Pullorum and *S*. Gallinarum are animal host-adapted *Salmonella* species in chicken and turkey. Some strains mainly produce infection in animals but could also affect human, e.g. *S*. Dublin in cattle, *S*. Choleraus in swine (7, 55).

Food borne illnesses are often time caused by non-typhi *Salmonella* species. This group includes over 2,500 serotypes that are found in the gastrointestinal tracts of birds,

mammals, reptiles and insects (55). Meats and eggs has been considered for long time to be the principal vectors for transmission, but cheddar cheese, ice cream, milk and milk powders, pasta, peanut butter, chocolate, and more recently cantaloupes, tomatoes, alfalfa sprouts, spices have caused salmonellosis as well (7,55). As the food chain becomes more integrated and the food chain expands further many other food items will be involved in cases of salmonellosis (6).

B. *Taxonomy*

Salmonella belongs to the family Enterobacteriaceae (35).The genus *Salmonella* contains two species; *S. enterica* and *S. bongori*, which was formerly subspecies V. Six subspecies are differentiated within *S. enterica* based on their biochemical and genomic characteristics, a Roman numeral and a name are used for the designation of these six subspecies as follows: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae* ; IIIa,*S. enterica* subsp. *arizonae* ; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*, and VI, *S. enterica* subsp. *indica*) (10). With regard to food safety *S. enterica* subsp. *enterica* is the subspecies of most concern because the strains within these serogroups are known to cause 99% of *Salmonella* infections in humans (6,10).

Table 1: Species and subspecies in the *Salmonella* genus (16)

***Salmonella* species**	**Subspecies**	**Number of Serovars**
S. enterica	enterica	1,478
	salamae	498
	arizonae	94
	diarizonae	327
	houteane	71
	indica	12
S. bongori		21
Total		**2,501**

By newer convention, names are retained only for subspecies *enterica* serovars, and these names are no longer italicized. The first letter is a capital letter "*S*" followed by the serovar names of subspecies *enterica* (e.g. Typhimurium or Montevideo). At the first citation of the serotype the genus name is given followed by the word " serotype" or the abbreviation "ser." Followed by the serotype name. This project follows the abbreviated modern naming system, i.e. *S.* Typhimurium rather than the more complete nomenclature *S. enterica,* subsp. *enterica* serovar Typhimurium. (10,55). The antigenic formulae are also used to name *Salmonella* serotypes. This designation includes: (i) subspecies designation (subspecies I trough VI), (ii) O (somatic) antigens followed by a colon, (iii) H (flagellar) antigens (phase 1) followed by a colon, and (iv) H antigens (phase 2, if present) i.e. *Salmonella* serotype IV 45:g,z_{51}:- (10) .The nomenclature detailed above is internationally accepted based on recommendations of the WHO Collaborating Center (55).

Table 2: Antigenic formulae of some *Salmonella* serotypes (16)

Serotype	Serogroup	Somatic antigen (O)	Flagella (H) antigens	
			Phase 1	Phase 2
S. Paratyphi A	A	1,2,12	a	(1,5)
S. Typhimurium	B	1,4, (5),12	i	1,2
S.Agona	B	4,12	f,g,s	-
S. Derby	B	1,4, (5),12	f,g	(1,2)
S. Typhi	D	9,12, (Vi)	c	1,2
S. Enteriditis	D	1,9,12	g,m	(1,7)

C. *Features*

Salmonella are facultative anaerobic, gram negative, small rods, motile (7,55). Temperature for growth ranges from 8°C to 45°C, strains can stand pH between 4 to 9, and is able to grow at water activities above 0.94. *Salmonella* is heat labile so the organism can be inactivated at ordinary cooking temperatures (> 70 °C) although the cooling time and values for temperature and time could change depending on the serotype and the food matrix. In addition *Salmonella* has been shown to tolerate up to 20% salt concentration (7,35). Under freezing conditions (from -23°C to -18°C) this microorganism is able to survive as long as seven years (7). The difficulty in controlling *Salmonella* is due to its ability to survive extreme environmental conditions (35).

The biochemical characteristics of *Salmonella* indicate that they are able to reduce nitrates to nitrites, produce gas from glucose (not always), produce hydrogen sulfide on triple-sugar iron agar, and they are usually able to use citrate as the sole carbon source (7,55). *Salmonella* can be further subdivided by phage typing, this method in conjunction with serotyping, pulse field electrophoresis (PFGE), determination of

antibiotic resistance patterns and plasmid profiling are methodologies that provide significant information for the assessment of *Salmonella* prevalence and epidemiology (55).

D. *Serological Identification (Kauffman-White scheme)*

The scheme used worldwide for serological identification of *Salmonella* serovars was first proposed by White and expanded by Kauffman (46). The list of 2,501 *Salmonella* serotypes is maintained and annually updated by the World Health organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (10). The Kauffman- White scheme (KW) is based on the antigenic structure of *Salmonella* serotypes (38). The antigenic properties and variations of the O (surface polysaccharide) and H (flagellar) antigens from each serovar are summarized and described in what is known as the antigenic formulae (58,79).

The structure of each microbial cell is dependent of a variety of antigenic molecules, which are at the time dependent of many determinant groups (chemical groups). Thus it is the chemical make up and the arrangement of these determinant groups what assign the immunological specificity of the antigen (35).

The cross absorption of antisera is used to reveal the antigenic structure of *Salmonella* (38). The composition and structure of polysaccharides, which constitute a part of the structure of the cell surface, allow for recognition and differentiation of O antigens (35). In the KW scheme O antigens are indicated in brackets when they are easily modified by mutation, otherwise they are underlined when these factors are determined by bacteriophages or plasmids (38). H antigens are present in the flagella, they are

composed of protein subunits called flagellin, that are typically diphasic and thought to help the bacteria to survive host immune responses (38). A capsular polysaccharide is found in some serovars (Typhi, Paratyphi C and Dublin) is termed “The virulence (Vi) antigen”. This factor first needs to be heated at 100 °C for 60 min to remove the capsule, otherwise it would not be agglutinable with anti-O antiserum (38).

Serological typing of *Salmonella enterica* serovars requires, over 150 O and H antigens and more than 250 antisera (14, 79). The problem with this conventional method is that it is laborious, time consuming, and cannot differentiate within serovars (62). It also depends on the availability of hundreds of antisera, needs highly trained personnel, consumes high volumes of reagents, and a minimum of three days is required to identify a serotype (1,14,80).

E. *Salmonella Infection*

Infections caused by *Salmonella* serotypes can produce enteric fever, gastroenteritis, and bacteremia or septicemia conditions (35, 57). *Salmonella* Typhi and Paratyphi are responsible for causingenteric fever (35). The period of incubation for this infection ranges from 8 to28 days and the common symptoms include fever, diarrhea, abdominal pain, headache (57). The antibiotics of choice for treatment of enteric fever are chloramphenicol, ampicillin or trimethoprim-sulfamethoxazole (57). When the infection is due to the consumption of a food item contaminated with non-typhoid *Salmonella* strains, the disease is often self-limiting in healthy individuals. Symptoms appear 8 to 72 hours after ingestion, and are less severe than in the previous case, and non-bloody diarrhea and abdominal pain disappear within 5 days. The treatment is based more on

fluid and electrolyte replacement than on antibiotic use. Infections caused by non-typhoid *Salmonella* serotypes can also evolve into systematic infections followed by chronic conditions (57).

Salmonellosis occurs when the bacteria have been able to survive the low pH in the stomach and reach the mucosa in the small intestine in adequate numbers to cause infection. Epithelial cells localized in the mucose midlayer are responsible of cover completely the *Salmonella* cells, which drive an inflammatory response. (35). The infection could progress to acute levels, depending on the serotype causing the illness (35).

F. *Salmonella Detection*

There are four steps for the recovery of injured *Salmonella* cells from a food matrix. First the pre-enrichment, where buffered peptone water or lactose broth can be used, followed by growth on a non-selective broth. This is followed by enrichment in selective broth, such as Rasspaport- Vasilliadis (RV) broth, Selenite Cysteine Broth (SC), or tetrationate broth (TT). Finally the subsequent isolation is done on selective Brillant green agar, Bismuth sulfite agar, Hektoen agar (HA) or XLD (55).

Some strains of *Salmonella* could have a different reaction to the combinations of inhibitory substances, incubation temperatures, selective enrichment broths and media (17). Some *Salmonella* serotypes (*S*. Anatum, *S*. Tennessee, *S*. Newington and S. Senftenberg) are lactose positive cultures (6) , for that reason it is important not to rely only on lactose to distinguish *Salmonella* from other microorganisms present in the food

matrix, but to utilize alternative selective media such as Mannitol Lysine Crystal Violet Brillant Green (MLCB) or Bismuth Sulphite Agar (6).

There are a wide variety of methods commercially available for *Salmonella* detection and identification. These include the use of antibodies to *Salmonella* antigens (Enzyme-Linked Immuno-sorbent assay (ELISA), immuno-chromatography, chemiluminescent immunoassay, antibody coated dipsticks, latex agglutination), electrical conductance methods, and polymerase chain reaction (PCR) (6,55). The principle limitation is that all of these techniques need a pre-enrichment step to reach detectable numbers of cells in the sample (10^4 -10^5 cells/ml). This factor makes it very difficult to develop a truly rapid method for detection and identification of *Salmonella,* that would allow to process the sample in a normal 8-hours work day (7, 55).

Food safety objectives from regulatory agencies encourage food industry manufacturers to establish full identification of *Salmonella* serotype to assist with traceability in the food processing (55). In addition, the capability to serotype or fingerprint is of importance for surveillance, inspection, and investigation of outbreaks. *Salmonella* subtyping can be accomplished by biotyping, phage typing, antibiotic resistant patterns, pulse field gel electrophoresis, and ribotyping (5).

The development of DNA-based methods for detection of *Salmonella,* have allowed for novel approaches in this field. The foundation of these methods is the hybridization of two complementary single –stranded molecules (one in the form of a probe, primer, DNA fragment or oligonucleotides developed in the laboratory and the other strand

corresponds to the target microorganism) to obtain double- stranded nucleic acid molecules under defined physical and chemical conditions.

Other diagnostic tools for *Salmonella* are the DNA microarrays. These are biochips, which enables hybridization by the presence of immobilized oligonucleotides to a solid base. Results can be analyzed automatically with use of an appropriate device (55). Numerous probes can be placed on a DNA chip and that number is expanding because of the continued growth of fully sequenced organisms (55).

II. Premi®Test *Salmonella*

A. *Principle*

Alternative strategies to replace or complement traditional serotyping have been proposed. These include ribotyping, ribosomal DNA intergenic spacer amplification, random amplification of DNA polymorphism, IS200 analysis, real-time PCR , amplified fragment length polymorphism, sequence analysis, and multiplex PCR (45), DNA microarrays (45, 66), and protein based methodologies have also been studied (14). High cost per sample, the necessity of specialized equipment and experienced personnel are the limitations commonly linked with these strategies.

The *Premi®Test Salmonella* system uses a methodology called multiplex ligation detection reaction (LDR) to generate a collection of circular DNA molecules which are subsequently PCR amplified (79).The test uses 25 DNA markers, three of which are generic markers used to verify that the isolate belongs to the *Salmonella* genus; once these generic markers have confirmed the presence of *Salmonella,* the other 22

remaining markers are used to identify the serotype. The system creates a specific hybridization profile for each *S. enterica* serovar. A profile is generated by detecting positive hybridizations, each of which generates a spot. Each spot has a certain value assigned, thus the Genovar score is determined by adding up the spots in the pattern that those spots have formed (16). Once a certain serotype yields a specific genovar score at least three independent times, this serotype-genovar score is added to the PTS database and the software will indicate the serotype as well. In cases where the serotype-genovar association has not been found often enough, the software will only indicate the genovar score. However, the genovar score can still be useful in traceability.

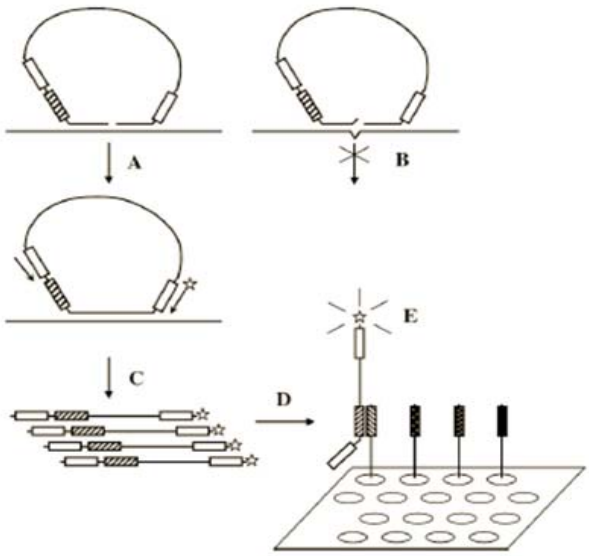
The system allows processing three samples in one single tube because of the use of unique ZIP codes assigned to each LDR probe which are complementary to the oligonucleotides (cZIPcodes) immobilized in the microarray (79).

The *Premi®Test Salmonella* (PTS) serotyping system is a promising tool for rapid identification of *Salmonella* serotypes. The PTS is a DNA-based method that allows processing of samples within 9 hours with no need of highly trained personnel to perform the test. In addition, the chances of contamination are reduced. These could provide advantages over the traditional Kauffman-White method which is typically viewed as the gold standard for *Salmonella* serotyping. Rapid identification of *Salmonella* serotypes could potentially assist meat companies, the Food Net surveillance system, and government agencies in tracing sources of contamination, thus allowing for rapid corrective action when needed. A major outcome would be the decrease in the number of *Salmonella*-contaminated products reaching the consumer.

B. ***Primers and ligation probes***

The foundation of this method is the detection of species-specific and serotype-specific nucleotide sequences (probes), this nucleic acids have been selected from DNA, rRNA, tRNA, mRNA, total RNA and tmRNA(dual tRNA-like and mRNA-like nature; also known as 10Sa RNA or SsrA) (2). Multiplex ligation detection reaction (LDR) is performed on the nucleic acids extracted from the target organism by providing a pair of probes; each probe has two oligonucleotides, which hybridize to adjacent target sequences. Then a thermo stable ligase is used to ligate the two parts of hybridized probes (2, 72,79). In one of the nucleic acid probes a unique region (ZIP codes, 20-25 nucleotide-long sequence) is placed between the target specific sequence and the primer binding section, this region is complementary to a corresponding region immobilized in the microarray (cZIP codes). Figure 1 describes the principle of this new methodology. The sequences for serotype-specific probes are protected by the manufacturer but the PCR primers had the following sequences corresponding to Primer Eco and Primer Mse respectively: 5′Biotin-GTAGACCTGCGTACCAATTC-3′ and 5′-GACGATGAGTCCTGAGTAA-3′ (79).

Figure 1: Principle of the Premi ®Test Salmonella serotype system (A) When properly hybridized to a target sequence, the nick lying between two adjacent LDR probe arms is ligated, so that a single circular fragment is generated. (B) Critical mismatches in the target sequence will cause ligation to fail, leaving the probes ends apart. (C) Successful ligation products are amplified by PCR using a single pair of amplimers annealing to complementary oligonucleotides (c ZIP codes, reverse –hashed box) spotted on the microarray. (E) Detection occurs thanks to a biotin label incorporated at the 5′- end of one of the PCR primer. The system can be multiplexed with many different LDR Probes, each bearing a unique ZIP code (black –filled boxed). The successive reactions are processed in a single tube (79).



C. *Microarray design and reading*

DNA -microarray technology represents a useful complement to current techniques for the characterization of serovars and strains based on differences and changes in their genetic content (24). The PTS microarray is designed in a manner that allows assessment of each critical point in the process. As detailed later in Table 6 ligation specificity and efficiency, PCR amplification, hybridization, label detection and quality can be evaluated. Inside this microarray there are unique oligonucleotides (cZIP)

immobilized, which find their complementary region (ZIP code) located on each LDR probes (2, 79). Results from the PTS system are read on a single-channel ATR03 reader consisting of a CCD-based transmission detector, connected to a standard computer, and the images are analyzed by using the Check Points software supplied by the manufacturer, this software reads the profile and provides the *Salmonella* serotype name as a final result (79). The spots in the profile are an outcome of positive and negative hybridizations, which generate a 14-digit code corresponding to a particular *Salmonella* serotype. This code is then translated to an exclusive identifier by means of the following mathematical algorithm: $\sum_{x=1}^{14} 2^{x-1}k$ (2).

The system is currently capable of identifying 100 *Salmonella* serotypes (Table 3). However, the identification capacity of the whole system can be expanded by inclusion of more probes targeting more *Salmonella* serotypes and by changing the specific sequences of the regions spotted in the microarray (2).

Table 3.- Premi ®Test *Salmonella* Serotypes

1, 4, [5], 12:i:-	Choleraesuis	Idikan	Mikawasima	Reading
Aberdeen	Coeln	Indiana	Minnesota	Regent
Abony	Colindale	Infantis	Monschaui	Rissen
Adelaide	Corvallis	Isangi	Montevideo	Saintpaul
Agona	Cubana	Jangwani	Muenchen	Sandiego
Albany	Derby	Javiana	Muenster	Schwarzengrund
Altona	Dublin	Kedougou	Napoli	Senftenberg
Anatum	Duisburg	Kentucky	Newport	Stanley
Banana	Eboko	Kottbus	Ohio	Stourbridge
Bareilly	Enteritidis	Lexington	Oranienburg	Telelkebir
Berta	Gallinarum Gallinarum	Lille	Orion	Tennessee
Blockley	Gallinarum Pullorum	Litchfield	Oslo	Thompson
Bovismorbificans	Give	Liverpool	Ouakam	Typhi
Braenderup	Gloucester	Livingstone	Panama	Typhimurium
Brandenburg	Goldcoast	London	Paratyphi A	Urbana
Bredeney	Grumpensis	Manchester	Paratyphi B	Virchow
Carrau	Hadar	Manhattan	Paratyphi B v Java	Wandsworth
Cerro	Havana	Matadi	Paratyphi C	Weltevreden
Chandans	Heidelberg	Mbandaka	Pomona	Worthington
Chester	Ibadan	Meleagridis	Poona	Yoruba

Source: DSM Premi®Test (79).

III. Antimicrobial resistance

A. *Resistance is an emerging problem*

Resistance to antimicrobials and particularly multidrug resistance is an emerging problem in Enterobacteriaceae for developing and developed countries (74). Resistant microorganisms have emerged as a result of improper use of antibiotics in human health as well as in agricultural practices (44). For example, in United States it has been reported that most of the antibiotics produced are fed to farm animals as growth promoters and to obtain a better meat to feed ratio (32). In the pork and poultry industry low levels of bacitracin, chlortetracycline, erythromycin, lincomycin, neomycin, oxytetracycline, penicillin, streptomycin, tylosin or virginiamycin are administrated in each ton of feed (44). Over the time these low doses of antimicrobials confer the ability of microorganisms to evolve mechanisms of defense, therefore making themselves less susceptible to the effect of the drug and contributing to treatment failure. *Salmonella* has been widely documented to possess resistance to several antibiotics used in medical treatment. In fact antibiotic-resistant *Salmonella* accounted for an annual mortality estimate of 4,760 deaths in the U.S alone (44). Antibiotic resistance has an important social and economic impact, and there is a need for stronger scientific and public health efforts to better regulate, control and monitor the use and abuse of antimicrobials (38).

Salmonella Typhimurium and *Salmonella* Heidelberg are ranked first and second respectively in multidrug resistance and are among the most commonly-isolated serovars from non-clinical, non-human sources (67). Four different antimicrobial resistance patterns were found in a study where *Salmonella* Heidelberg isolates from swine were

tested for resistance to a panel of 12 antibiotics (67). All isolates showed resistance to amoxicillin-clavulic acid, amikacin, ceftrixone, ciprofloxacin, cephalothin, and gentamicin (67). *S.* Typhimurium and *S*. Muenchen isolates from swine have shown resistance to ampicillin, chloramphenicol, amoxicillin, clavulic acid, kanamycin, streptomycin, sulfisoxazole, and tetracycline (67). In poultry production, sulphamides (sulfisoxazole), fluoroquinolones (nalixidic acid) and tetracyclines (tetracycline) are currently used in many countries worldwide (15). *S*. Typhimurium and *S*. Enteritidis have been shown to be more antibiotic resistant than other serotypes commonly isolated from poultry sources (15).

Because of the concern over increasing resistance, the CDC, FDA-CVM, (Center for Veterinary Medicine) and USDA-FSIS (Food Safety and Inspection Service and Agricultural Research Services) established The National Antimicrobial Resistance Monitoring System (NARMS) for Enteric Bacteria to monitor antimicrobial resistance among foodborne enteric bacteria isolated from humans and foods (22). In 2006, the NARMS published a list containing the categories of antimicrobials of importance for human health (Table 4); antimicrobials in this list are classified based on whether the evaluated antimicrobial is unique or one of the few alternatives for treatment of human diseases (61).This report also details two multidrug resistant patterns. 5.5 % of non-Typhi *Salmonella* are thought to be resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole/sulfisoxazole, and tetracycline (ACSSuT) (61) . This percentage is lower than the 8.8% observed in 1996 due to the overall reduction of resistance in some serovars. However other *Salmonella* serovars appear to be acquiring resistance to these antibiotics, such as *S*. Newport. Evaluated in 2006, the resistance of

this serotype increased 6% from 1996. According to The CDC (2009), a second multidrug resistant pattern (ampicillin, chloramphenicol, streptomycin, sulfamethoxazole or sulfisoxazole, tetracycline, amoxicillin-clavulanic acid, and ceftiofur) was not detected in any serotype in 1996. In 2006, a 2% increase was observed among non- typhi *Salmonella* serovars (61).

Table 4: World Health Organization's categorization of antimicrobials of critical importance to human medicine (61)

	Categorization of Antimicrobials	**Subclass**
Critically Important	Aminoglycosides	Amikacin Gentamicin **Streptomycin**
	Aminopenicillins	**Ampicillin**
	β-Lactamase inhibitor combinations	Amoxicillin-clavulanic acid
	Cephalosporins (3rd generation)	Ceftriaxone*
	Ketolides	Telithromycin
	Macrolides	Azithromycin Erythromycin
	Quinolones	Ciprofloxacin **Nalidixic acid**
Highly Important	Aminoglycosides	Kanamycin
	Cephalosporin (1st generation)	Cephalothin
	Cephamycins	Cefoxitin
	Folate pathway inhibitors	Trimethoprim-sulfamethoxa
	Phenicols	**Chloramphenicol**
	Sulfonamides	Sulfamethoxazole Sulfisoxazole
	Tetracyclines	**Tetracycline**
Important	Lincosamides	Clindamycin

The categories provided by the WHO could serve as a guide to determine which antibiotics should constantly be observed because resistance to them will significantly

decrease the alternatives for medical treatment. Consequently more severe doses and longer periods of hospitalization are required.

B. *Mechanism of action of antibiotics*

Ampicillin is part of the beta- lactam antibiotics, and belongs to the penicillin group. Its main difference with other beta-lactams is the presence of an amino group. The interaction of penicillin-binding protein with the bacterial cell wall results in the disruption of synthesis of the bacteria cell wall (11).The principal mechanism for B-lactam antibiotic resistance is the acquisition or hyperexpression of β-lactamases (11, 74).

The use of chloramphenicol in the European Union and North America is exclusively for non-food-producing animals (34). Chloramphenicol blocks the formation of the peptide bond between amino acids by inactivating the peptidyltransferase reaction, and this mechanism of action makes chloramphenicol a highly effective protein synthesis inhibitor (74). Enzymatic inactivation by chloramphenicol acetyltransferase is the leading mechanism of resistance to chloramphenicol in both gram-positive and gram-negative bacteria (34,74). In addition, other mechanisms have been proposed and include: target site mutations, permeability barriers, phosphotransferase inactivation and some effux systems (74).

Nalidixic Acid was one of the first quinolone antibiotics developed. It is effective against both gram-positive and gram-negative bacteria by inhibiting a subunit of DNA gyrase. This has been demonstrated to be the mechanism of action of all antimicrobials

belonging to the quinolone group (27, 38). Antibiotic resistance occurs when an altered target is developed (38).

Streptomycin belongs to the aminoglycosides group. Amyglycosides target bacterial ribosome (30S unit) which results in the misreading of the genetic code during synthesis of protein resulting in growth inhibition (38, 40, 74). Modifying enzymes and reduction in uptake are the main resistance mechanism developed by bacteria resistant to this drug. (38).

Sulfisoxazole belongs to the sulfonamides group and these antimicrobials work by affecting the DNA, RNA and protein synthesis. The major factors responsible for the resistance are the permeability barrier and/or efflux pumps, natural insensitivity target enzymes and changes in the target enzymes (51)

Tetracycline inhibits microbial growth by inhibiting the elongation step of protein synthesis. When it forms a complex with a divalent cation in the cytoplasm, the antibiotic binds reversibly to the 16S ribosomal RNA (rRNA) of prokaryotes near the ribosomal acceptor A site, thus preventing binding to aminoacyl-tRNA to this site. Four mechanisms have been identified to confer resistance: i) energy- dependent efflux (this mechanism does not allow tetracycline to get into the cytoplasm); ii) tetracycline molecule inactivation; iii) rRNA mutations, and iv) ribosomal protection (74).

Tetracycline's active efflux is the major mechanism of bacterial resistance. Transporter proteins, located in the cytoplasmatic membrane, mediate energy-dependent efflux of the tetracycline, allowing tetracycline- resistant cells to lose the accumulated drug faster than susceptible cells do (51). These proteins are encoded by naturally occurring genetic

units which include all genes involved in resistance (tetracycline determinants). These genetic units confer resistance by removing tetracycline from the cytoplasmatic matrix (51).

Continuous monitoring of pathogens and their acquisition of antibiotic resistance genes is important to human health, but few laboratories testing for the presence of pathogens in food samples are able to provide this relevant information (29). Therapeutic options become limited when multidrug resistant pathogens are encountered, which constitutes an emerging public health issue worldwide (33).

IV. *Pulse Field Gel Electrophoresis (PFGE)*

Pulse Field Gel Electrophoresis (PFGE) is the gold standard for molecular typing of *Salmonella* (70). PFGE subtyping has been successfully applied to the subtyping of many pathogenic bacteria to establish the degree of genetic relatedness between isolates of the same species or serotype (59). This methodology has been valuable in tracking sources of outbreaks in epidemiological studies (3). PFGE has been repeatedly shown to be more discriminating than other methods such as ribotyping for many bacteria (21, 55,59,70).

Restriction enzymes that recognize few sites in the chromosome are used to generate large DNA fragments (55). These fragments are then separated by constantly changing the direction of the electrical field during electrophoresis (21). PFGE can separate DNA of different sizes using a determined switch time, which represents the duration of the alternating electric fields. There is a maximum size range related to each switch interval that does not allow further resolution (8).

Enzymes used for fingerprinting are chosen based on the length of recognition sequence of the enzyme and the GC content (69). For *Salmonella* fingerprinting *XbaI* (5′…T˅CTAGA…3′) is the enzyme of choice. When isolates require further characterization *BlnI* nuclease enzyme is also considered (48). PFGE using these two enzymes have provided good discriminatory power for identifying of sources of contamination (48).

Kaldhone et al. (2008) applied PFGE to characterize turkey isolates collected from different sources (41). To fully evaluate the isolates, *XbaI* and *BlnI* were used and 55 different patterns were identified from 180 isolates. The authors emphasized the importance of using a combination of enzymes to distinguish among closely related serovars (41).

PFGE with *XbaI* was useful to determine relatedness and genotypic changes of historic (1988-1995) and contemporary (1999-2001) isolates of *Salmonella* Newport (5). The same methodology was followed to identify the genomic DNA fingerprint profiles of *Salmonella* Heidelberg isolated from retail meats (81). By using this technique they found clones widely distributed in different types and brands of meats collected during 5 years from diverse retail stores (81).

PFGE is considered superior over other molecular typing methods (48). At each restriction site, 90% of the chromosome and approximately 0.05% of the genome is scanned, contributing to the high resolving power of the PFGE system (53). Nevertheless PFGE is not discriminatory enough for identification of some *Salmonella* serotypes that have been shown to have intracellular DNAse activity that degrades the

genomic DNA, such as *Salmonella* Panama (48). Addition of 50 µl of thiourea to the gel buffer solved this problem and obtained better resolution on the bands. (48).

The choice of the restriction enzyme and conditions for electrophoresis need to be optimized for each species (23, 59, 70). DNA restriction patterns generated by PFGE are stable and reproducible by different laboratories. The CDC provides nationally standardized procedures, proficiency testing programs, pattern databases and data sharing between State Federal and Labs via the Pulse –Net USA. PFGE was used in this project to generate a dendrogram that will make it possible to evaluate the genetic relatedness of serotypes obtained from different areas of the processing plant. In addition PFGE results would be used to determine genetic profiles of antibiotic-resistant *Salmonella* strains.

V. OBJECTIVES:

1. The objective of this project was to evaluate the use of the *Premi ®Test Salmonella* system as a serotyping tool to identify pork and poultry isolates obtained from vertically integrated operations and to compare the performance of the PTS system with traditional Kauffman-White (KW) serotyping methods.

2. To evaluate the antibiotic resistance of *Salmonella* isolates recovered from poultry and pork commercial sources and to study the genetic relatedness among these isolates.

CHAPTER II

MATERIALS AND METHODS

I. Evaluation and Performance of the Premi®Test Serotyping System

This first part of this study was conducted in association with Texas A&M University, who were responsible of sample collection and isolation of *Salmonella*. Mississippi State University performed the traditional serotyping on positive isolates which were compared against the results obtained at the University of Nebraska –Lincoln with the Premi®Test device.

A. Stored Isolates

Ninety *Salmonella* strains were obtained from the USDA–ARS-SPARC in College Station, TX, who generously allowed us to use them for this project. These cultures had been isolated using a modified version of the USDA method, serotyped according to the traditional Kauffman – White scheme, and cryogenically stored. Isolates were shipped to the University of Nebraska-Lincoln (UNL) for typing using the PTS system for comparison. An additional 10 cultures were obtained from cryogenically stored cultures in the UNL Food Processing Center Laboratory's stock culture collection for a total of 100 isolates.

B. Fresh Isolates

Fifty *Salmonella* strains from poultry and fifty from pork were isolated by investigators at Texas A&M using a modified version of the USDA method. Samples were collected from carcasses at different stages during the processing chain: live haul receiving, scalding, after evisceration, after chemical treatments, after cooling, and from final products. Following collection, samples were incubated overnight in buffered peptone

water and then transferred to tetrathionate and Rappaport-Vassiliadis broth. After incubation overnight at 42ºC, a loopful of the sample was streaked onto XLT4 and BGS agar. Samples showing typical colonies were screened for *Salmonella* using the GeneQuence® from Neogen (Lansing, MI). Samples with positive results for *Salmonella* from the GeneQuence® were confirmed using the API 20E biochemical system from BioMerieux. A subculture was then shipped to Mississippi State for serotyping according to the traditional Kauffman-White scheme, and to the University of Nebraska- Lincoln for typing by the *Premi ® Test Salmonella* system.

Sponge samples were collected as follows: Samples were taken by pre-moistening a dry, sterile cellulose sponge (HydraSponge®; 3M, St. Paul, MN) with 25 ml of Butterfield's buffer (3M, St. Paul, MN). Using a sterile plastic glove, the sponge was removed from the sterile sample bag, all excess buffer expressed into the bag, and the sponge firmly rubbed against the surface of the animal, hide, carcass, or equipment approximately 10 times in the horizontal and 10 times in the vertical direction in approximately a 100-cm^2 area. The sponge was then turned over and the swabbing of the sample area repeated. For smaller pieces (e.g. ears and feet) and offal, the entire piece was swabbed. After sampling, the sponge was placed back into the sterile sample bag containing the expressed buffer and labeled. Labeled sample bags containing the sponge samples were packed into a cooler with cold packs for transport to the Food Microbiology Laboratory, Department of Animal Science, Texas A&M University, College Station, TX. Samples obtained from outside Texas were shipped overnight for next day delivery. Upon arrival at the laboratory, the temperature of samples was recorded and the samples prepared for analysis.

C. Premi -Test Salmonella Procedure

The Premi-test *Salmonella* system is a commercialized kit containing two sets of reagents. The first group of reagents needs to be stored under frozen conditions (-20 °C) and it contains: i) small tubes with 10 ul of genomic DNA solution, ii) solution A, which is a mix made of ligation probes and thermostable DNA ligase, iii) solution B, that is an exonuclease , iv) Solution C, which contains a mix of PCR primers, deoxynuclesiode triphosphates and thermostable polymerase, and finally v) Peroxidase – conjugated streptavidin solution used as a biotin label.

The second group of reagents are stored at room temperature and this set contains: i) detection buffer ii) lysis buffer, iii) blocking buffer iv) staining solution, a peroxidase substrate (79).

The protocol consists of the following steps:

1. Sampling
2. Lysis
3. DNA recognition step A
4. DNA recognition step B
5. DNA recognition step C
6. Detection step

1. Sampling

- Samples were streaked for isolation on Tryptic Soy Agar plates (Acumedia- Neogen Corp.) incubated overnight at 37°C
- 100 µl of Lysis Buffer were dispensed into a 1.5 ml tube. A separate tube was used for sample

- A single colony was pierced through the agar till the bottom using the colony sampler
- Colony sample was then placed and twisted in the 100 µl Lysis Buffer. Tube was closed and vortexed

2. Lysis
    - The 1.5 ml tubes were transferred with the resuspended cells to a heating block (Thermo mixer) and incubated at 99 °C for 15 min at 400 rpm.
    - After 15 min the tubes were cooled down to room temperature by placing the tubes on the table, and vortexed before continuing
3. DNA recognition step
    - 5µl of solution A was added to each reaction tube of the strip (supplied with the kit). Next, 10 µl of DNA extract (from step 2) of each sample was added
    - Tubes were closed and spun down briefly using a minifuge to collect both sample and solution A at the bottom of the tubes. Tubes were mixed well by tapping against each strip, then spun down again.
    - Strips were placed in the PCR instrument and the CP step A program was run (total sample volume 18 µl)
4. DNA recognition step B
    - Solution B was prepared according to the pipetting scheme provided by the manufacturer, which included mixing solution buffer with appropriate amounts of solution B depending upon the number of

samples being tested. Tubes were the mixed by votexing and spinning down briefly

- After step A, tubes were spun down briefly and 15 µl of solution B was added to each sample in the strip(s). Tubes were closed, mixed by tapping each strip and spun down briefly
- Strips were placed in the PCR instrument and the CP step B program was run (total sample volume 33 µl)

5. DNA recognition step C

- Solution C was prepared according to the pipetting scheme provide by the manufacturer , mixed properly , vortex and spinning down briefly
- 15 µl of solution C was added to each sample in the strips, tubes were closed, and mixed by tapping each strip and spun down briefly
- Strips were placed in the PCR instrument and the CP step C program was run (total sample volume 48 µl)

Table 5: PCR profiles for Premi®Test *Salmonella*

CP step A	
Cycle 1 (1x): Cycle 2 (24x): Cycle 3 (1x):	3 min. at 95°C 30 sec. at 95°C 5 min. at 65 °C 2 min. at 98°C; ∞ (unlimited) at 4°C
CP step B	
Cycle 1 (1x):	45 min. at 37°C; 10 min at. 95°C; ∞ (unlimited) at 4°C
CP step C	
Cycle 1 (1x): Cycle 2 (35x): Cycle 3 (1x):	10 min. at 95°C 5 sec. at 95°C; 30 sec. at 55°C; 30 min. at 72 °C 2 min. at 98°C; ∞ (unlimited) at 4°C
CP melt	
Cycle 1 (1x):	2 min. at 95°C; ∞ (unlimited) at 4°C

6. Detection step

- 300 µl of Detection Buffer was added to each Array tube (one AT for each of three samples) followed by agitation in the Thermo mixer for 2 min (400 rpm) at 50°C
- The Detection Buffer was removed carefully and the previous step was repeated
- The Detection Buffer was replaced by 300 µl of fresh Detection Buffer and 10 µl of sample from each tube of one strip on step C was added to each AT. The total volume of each AT was 330 µl
- Lids were closed and AT's were shaken for 30 min at 50°C
- After 30 min, the Detection Buffer was replaced by 300 µl of Blocking Buffer; the AT(s) were shaken for 5 min at 50 °C (400 rpm)
- The Blocking Buffer was replaced with 300 µl of fresh Blocking Buffer. The temperature in the Thermo mixer was adjusted to 30°C and ATs were incubated for 10 min while the Themo mixer cooled down from 50°C to 30°C
- The Conjugate solution was prepared according to the pipetting scheme.
- The Blocking buffer was removed and 150 µl of Conjugate dilution were added and incubated for 15 min at 30°C (400 rpm)
- The Conjugate solution was removed from ATs and 600 µl of Detection buffer were added, tubes were shaken for 2 min at 30°C (400 rpm)
- The Detection Buffer was replaced with 600 µl of fresh Detection Buffer and tubes were shaken for 2 min at 30°C (400 rpm)

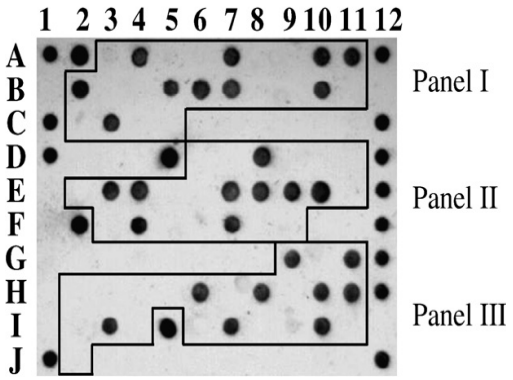
- The Detection Buffer was removed and 150 µl of Staining solution was added to each AT and incubated for 15 min at room temperature
- After 15 min AT were read using the check points software and reader provided by the manufacturer

Table 6. Probe types and capture position on the DNA microarray

Probe Target	Panel	Array- Tube	Comments
Detection control	None [a]	A1 A12 C1 C12 D1 D12 E12 F12 G12 H12 J1 J12	Biotinylated oligonucleotides spotted on the microarray and used as control spots for the staining process. Reference spots for the image analysis software.
Hybridization control probes	None [a]	A2 D5 F2 I5	Biotinylated probe with blocked 3'-end complementary to spotted oligonucleotide at indicated array positions
Salmonella general LDR	Panel I Panel II Panel III	B7 B10 C3 E10 F4 F7 I3 I7 I10	This LDR probes bind to ubiquitous highly *Salmonella* sequences such as *inv A* and other conserved sequences
Negative LDR controls	Panel I Panel II Panel III	B9 C2 C5 F3 F6 F9 I6 I9 J2	These LDR probes match the *Salmonella* general LDR probes but contain annealing mismatches aimed at adjusting the sensitivity threshold.
DNA controls	Panel I Panel II Panel III	B8 B11 C4 E11 F5 F8 I4 I8 I11	These LDR probes bind to a control (non- *Salmonella*) sequence spiked at suboptimal concentration on the ligation mix and yield detectable signals only in case of insufficient genomic DNA concentration.
LDR typing probes	Panel I Panel II Panel III	A3-11 B2-6 D6-11 E2-9 G9-11 H1-10 I1	These LDR probes bind to critical *Salmonella* markers used to infer the serovar signature

[a] Out of panel position (79)

Figure 2. Typical DNA microarray picture obtained with the ArrayTube®.



This format uses a DNA microarray fixed at the bottom of a micro-reaction vial. The microarray consists in unique complementary (cZIP) oligonucleotides targeting individual LDR probes. When hybridization of the PCR-amplified ligation products to the microarray is complete, colorimetric detection of the positive reactions is initiated. Polygons delineate panels in the array. Each panel defines the typing results of one strain and consists in control spots and specific marker spots (listed in Table 2). The strains typed in this figure belong to serovar Enteritidis (Panel I), Hadar (Panel II) and Infantis (Panel III) (79).

II. Antimicrobial Resistance Test

One hundred fresh isolates collected from pork and poultry commercial sources were tested for antibiotic resistance using the HardyDisk™ Antimicrobial Sensitivity Test (ATS) (Hardy Diagnostic, Santa Maria, CA).This method is based on the Kirby – Bauer procedure, an agar diffusion test, for semi-quantitative *in vitro* susceptibility of rapidly growing bacterial pathogens (37). HardyDiskTM AST Disks are prepared by saturating high-quality 6mm diameter white filter paper disks with accurately determined amounts of antimicrobials. Isolates were evaluated for resistance to a panel of six antibiotics (streptomycin, ampicillin, nalixidixic acid, chloramphenicol, sulfisoxazole and tetracycline), which were selected based on the categorization of antimicrobials of critical importance published by The World Health Organization (Table 10), and results

obtained in several studies (15; 67) which show increase on resistance to one or more antibiotics on serotypes associated with poultry and pork.

The antimicrobials analyzed and their disk potencies were as follows: ampicillin (10 µg), chloramphenicol (30 µg), nalixidic acid (30 µg), streptomycin (10 µg), tetracycline (30 µg), sulfixozasole (250 µg).

Isolates were grown overnight from frozen stocks in Tryptic Soy Agar (Acumedia, Neogen Corp.), and incubated at 37 °C. Colonies were then transferred to Tryptic Soy broth and the concentration was adjusted to 0.125 absorbance using a wavelength of 550 nm. Once the concentration was adjusted the cultures were swabbed onto Mueller Hilton Agar, covering the surface of the plate three times. The Hardy disks were then placed on the swabbed plate and incubated at 37 ° C for 18 hours. The zones of inhibition were read the next day using the Flash & Go automated-counter and results were assessed according to the HardyDisk™ manufacturer's recommendations (Table 7).

Table 7.- Disk Diffusion Zone Diameter Chart

Antibiotic	Resistant (mm)	Intermediate (mm)	Susceptible (mm)
Ampicillin	≤13	14-16	≥17
Chloramphenicol	≤12	13-17	≥18
Nalixidic Acid	≤13	14-18	≥19
Streptomicin	≤11	-	≥15
Sulfixozasole	≤12	13-16	≥17
Tetracycline	≤11	-	≥15

III. Pulse-Field Gel Electrophoresis Procedure

The standardized CDC laboratory protocol for Molecular subtyping of non-typhoidal *Salmonella* serotypes and the CHEF –DR III pulsed gel electrophoresis system from Bio-Rad were used in this study. The formulations for solutions needed for the procedure are detailed in appendix A. The CDC- PFGE procedure has three major steps i) preparation of plugs and cell lysis ii) restriction digestion of DNA with *XBaI* iii) preparation and loading of agarose gel. These steps were performed as follows:

i) Preparation of Plugs and cell lysis

1. Cells were grown on TSA plates and removed using a sterile cotton swab
2. Cell were suspended in 9 ml of cell suspension buffer and the optical density was adjusted to 1.3-1.4 (610 nm wavelength)
3. 400 µl of adjusted cell suspension was transferred to a 1.5 ml centrifuge tube containing 20 µl of Proteinase K from Fisher Bioreagents (20 mg/ ml stock) and mixed gently by pipetting up and down with 400 µl of melted 1% Seakem Gold agarose
4. The mixture was then dispensed into the wells of a reusable plug mold and allowed to solidify at room temperature for 10-15 minutes
5. Plugs were then transferred to a 50 ml tube containing 5 ml of cell lysis buffer and 25 ul of Proteinase K. Tubes were incubated overnight at 54°C in a shaker incubator (150-175 rpm)
6. Tubes were removed from the shaker incubator and the cell lysis buffer/ Proteinase K mixture was removed and 10 ml of pre-warmed (50°C) ultrapure water was added

7. Tubes were returned to the shaker incubator for 10-15 minutes at 50°C.
8. The water was removed and the previous step was repeated one more time.
9. The water was removed and 10 ml of pre-warmed (50°C) TE buffer were added, tubes were placed in the shaker incubator for 10-15 minutes at 50°C
10. The TE buffer was poured off and the previous step was repeated three more times
11. The last wash was removed from the tube and 10 ml of sterile TE Buffer was added. Plugs were kept at 4°C until needed for restriction digestion of DNA with *XbaI*

ii) Restriction Digestion of DNA In Agarose Plugs with *XbaI*

1) A 2.0-to 2.5-mm-wide slide was cut from the agarose plug of each isolate as well as from the *S*. Branderup H9812 standard.
2) Plugs were transferred to a 1.5 ml centrifuge tube containing 200 µl of 1X restriction buffer .
3) Tubes were incubated overnight at 37 °C.

iii) Preparation and loading of gel in the PFGE system

1) Restriction buffer was replaced with 200 µl of 0.5 TBE and incubated at room temperature for 5 minutes.
2) The plugs were removed from the tube and placed on the comb; plugs were sealed to the comb by adding melted 1% Seakem agarose gel to the ends.
3) While the gel solidified 100 ml of melted 1% Seakem agar were poured into the assembled gel casting box and 2000 ml of 0.5 X TBE was added to the electrophoresis chamber and cooled to 14 °C.

4) The comb was removed and the wells were filled with melted 1% Seakem agarose gel; the gel was then placed into the PFGE chamber.
5) Electrophoresis settings for the CHEF DR –III were as follows :

    Initial switch time: 2.2s

    Final switch time: 63.8 s

    Voltage: 6V

    Included Angle: 120 °

    Duration: 19 hours
6) After 19 h, the gel was removed from the box and stained for 30 minutes with ethidium bromide (10 mg/ml). The gel was washed every 20 minutes for 1 hour with 500 ml ultrapure water.

CHAPTER III

RESULTS

I. Premi ® Test *Salmonella*

A. *Culture collection*

A total of 100 isolates from the USDA and UNL culture collections were tested using the PTS system and compared to the traditional Kauffman-White (KW) scheme. The results from these tests are shown in Tables 8 through 11. Table 8 shows a comparison of KW versus the PTS system on serotypes isolated from **poultry** that were **not present** in the PTS database. The PTS system did not match KW serotyping on all 27 *Salmonella* serotypes that were tested. The system did respond with either a Genovar score or an alternative serotype, and correctly identified the isolates as *Salmonella* species 96% of the time. Table 9 shows the comparison of KW with the PTS system on serotypes isolated from **poultry** that **were present** in the PTS database. The PTS system matched KW serotyping on 49% of isolates tested. Again, the system did respond with either a Genovar score or an alternative serotype, and correctly identified the isolates as *Salmonella* species 95% of the time.

Table 10 shows a comparison of KW versus the PTS system on serotypes isolated from **pork** that were **not present** in the PTS database. Of the five that were tested, none matched the KW serotyping results. The system was able to correctly identify all isolates as *Salmonella* species, and produced either a Genovar score or alternative serotype. Table 11 shows the comparison of KW serotyping with the PTS system on serotypes isolated from **pork** that **were present** in the PTS database. The PTS system matched KW serotyping on 76% of the isolates tested. For the remaining isolates, a

Genovar score or an alternative serotype was produced. The system also correctly identified all 28 isolates as *Salmonella* species.

Table 8: Comparison of Kaufmann-White (KW) and PTS results from USDA isolates collected from POULTRY, NOT PRESENT in the PTS database

KW	PTS Results
S. G22-,23+	Genovar 3171
S. Bere	Genovar 3303
S. 4, 12:i:-	Genovar 3997
S. 4,12:-:1,2	Genovar 13487
S. 4,5:2:-	*S*. Typhimurium
S. 4,5:d:-	*S*. Schwarzengrund or Grupensis
S. 4,5:i:-	*S*. Typhimurium
S. 6,7: nonmotile	Genovar 7604
S. 6,7:-:1,5	*S*. Muenster or Montevideo
S. 6,7:-:1,6	Muenster or Reading 14958.F
S. 6,7:k-	*S*. Brandenburg
S. Alachua	*S*. Cubana
S. Cape	*S*. Thompson
S. Essen	*S*. Derby
S. Fresno	*S*. Ouakam or Meleagridis
S. Gaminara	*S*. Typhimurium
S. Kiambu	Genovar 15533
S. Menston	*S*. Oranienburg
S. Mississippi	Genovar 16013
S. Molade	Genovar 10299
S. Norwich	Genovar 3104
S. Remo	*S*. Schwarzengrund or Grupensis
S. roughO:y:1,7	*S*. Pomona
S. Thomasville	*S*. Orion
S. Truro	*S*. Typhimurium
S. Try Z29	No Salmonella
S. Uganda	Genovar 13487
Total Match	0/27 (0%)
Salmonella species confirmed	26/27 (96%)

Table 9. Comparison of Kaufmann-White (KW) and PTS results of USDA isolates collected from POULTRY, *PRESENT* in the PTS database.

KW	PTS Results
S. Havana	Genovar 3171
S.1,4,5,12:i:-*	*S*. 1,4,(5),12:I
S.1,4,5,12:i:-*	*S*. 1,4,(5),12:I
S. Agona	*S*. Montevideo
S. Blockey*	*S*. Blockey
S. Braenderup*	*S*. Braenderup
S. Braenderup*	*S*. Braenderup
S. Colindale	*S*. Montevideo
S. Cubana*	*S*. Cubana
S. Derby*	*S*. Derby
S. Enteriditis	*Salmonella* suspected
S. Enteriditis	*S*. Hadar
S. Enteriditis	*S*. Heidelberg
S. Enteriditis*	*S*. Enteriditis
S. Hadar*	*S*. Hadar
S. Heidelberg*	*S*. Heidelberg
S. Infantis	*S*. Heidelberg
S. Kentucky	Genovar 10299
S. Kentucky	No Salmonella
S. Lille	Genovar 14537
S. Litchfield	*S*. Ouakam
S. Livingstone	*S*. Lille
S. Meleagridis*	*S*. Meleagridis
S. Montevideo*	*S*. Montevideo
S. Muenchen*	Montevideo or Muenchen
S. Muenster	Genovar 14948
S. Oranienburg	*S*. Monschaui
S. Orion,var, 15, 34*	*S*. Orion
S. Quakam*	*S*. Quakam
S. Senftenberg*	*S*. Senftenberg
S. Stanley	*S*. Muenchen
S. Schwarzengrund	Serovar cannot be identified
S. Tennessee	*S*. Ouakam
S. Thompson*	*S*. Thompson
S. Thompson*	*S*. Thompson
S. Typhimurium*	*S*. Typhimurium
S. Typhimurium	Genovar 2098
S. Typhimurium	No Salmonella
S. Worthington	Genovar 14377
Total Match*	19/39 (49%)
Salmonella species confirmed	37/39 (95%)

Table 10: Comparison of Kaufmann-White (KW) and PTS results from PORK, *NOT PRESENT* in the PTS database

KW	PTS
3,10:L,W-Monophasic	*S*. Meleagridis
S. Johannesburg	*S*. Urbana
S. Menhaden	*S*. Give
S. New Brunswick	*S*. Give
S. Uganda	Genovar 13487
Total	0/5 (0%)
Salmonella species confirmed	5/5 (100%)

Table 11: Comparison of Kaufmann-White (KW) and PTS results from PORK, *PRESENT* in the PTS database

KW	PTS
1,4,5,12:I-*	*S*. 1,4,5,12:i
S. Agona*	*S*. Agona
S. Anatum*	*S*. Anatum
S. Braenderup*	*S*. Braenderup
S. Derby*	*S*. Derby
S. Havana	Genovar 9610
S. Heidelberg*	*S*. Heidelberg
S. Heidelberg*	*S*. Heidelberg
S. Infantis*	*S*. Infantis
S. Javiana*	*S*. Javiana
S. Livingstone	Genovar 14537
S. Mbandaka*	S. Mbandaka
S. Meleagridis*	*S*. Meleagridis
S. Montevideo	*S*. Schwarzengrund or Grupensis
S. Muenchen	*S*. Newport
S. Muenster*	*S*. Muenster
Multiple Serotypes*	*S*. 1,4,5,12:i
S. Newport*	*S*. Newport
S. Orion*	*S*. Orion
S. Schwarzengrund*	*S*. Schwarzengrund or Grupensis
S. Tennessee	Genovar 56
S. Thompson*	*S*. Thompson
S. Typhimurium*	*S*. Typhimurium
S. Typhimurium*	*S*. Typhimurium
S. Typhimurium*	*S*. Typhimurium
S. Typhimurium*	*S*. Typhimurium
Untypable	*S*. Meleagridis
S. Urbana*	*S*. Urbana
S. Worthington	*S*. San Diego
Total Match*	22/29 (76%)
Salmonella species confirmed	29/29 (100%)

B. *Fresh isolates*

A total of 100 fresh isolates (50 from poultry, 50 from pork) were tested using the PTS system and compared to the Kauffman-White (KW) serotyping method. The results from these tests are shown in Tables 12 and 13. Table 12 shows a comparison of KW versus the PTS system on fresh isolates collected from **poultry** operations. The first column indicates the *Salmonella* serotyping result from the KW method, while the second column indicates the number of isolates of that serotype that matched the PTS system. The third column shows the locations that matching isolates were collected from. The last two columns show the alternative identifications produced by the PTS system and the locations where these isolates were found. The dominant serotype isolated was *S*. Braenderup, which comprised 52% of the total number of serotypes. Of these the PTS system matched the KW method in 78% of the isolates. The total match rate was 60% for all isolates. For those isolates that did not match, the system responded with either a Genovar score or an alternative serotype. The system also correctly identified the isolates as *Salmonella* species 100% of the time.

Table 13 shows a comparison of KW versus the PTS system on fresh isolates collected from **pork** operations. The information is outlined in the same format as Table 8 described above. The dominant serotype isolated was *S*. Anatum, which comprised 28% of the total number of serotypes. Of these the PTS system matched the KW method in 73% of the isolates. The total match rate was 66% for all isolates and the system correctly identified the isolates as *Salmonella* species 100% of the time. Again, for those isolates that did not match, the system responded with either a Genovar score or an

alternative serotype. One that was unknown by the KW method was given a Genovar score (7540) by the PTS method.

Table 12: Comparison of Kaufmann-White (KW) and PTS results of fresh isolates collected from POULTRY

Salmonella serotype (KW)	Salmonella PTS RESULTS			
	Complete Match	**Location**	**Other I.D. (# Isolates)**	**Location**
S. Braenderup	26	Carcass, rinse feathers on, outside beetle, inside beetle, soil inside, soil outside, water, feed, ceca, booty, beetle, larvae	Genovar 9614 (2)	Ceca, litter
			Genovar 9646 (1)	Water
			Genovar 11658 (1)	Soil inside
			Manhattan (2)	Inside beetle, booty
			Unidentified (1)	Feed
S. Kentucky	0		Genovar 10299 (6)	Scalder, live chicken loader, chicken after picking
			Genovar 102983 (1)	Chicken feet
			Genovar 14907 (1)	Live chicken loader
			Genovar 15423 (1)	Feet chute
			Ohio (1)	Inedible barrel evisceration
			Unidentified (1)	Chicken after picking
S. Newport	3	Carcass rinse feathers on	Genovar 13502 (1)	Booty
S. Anatum	1	Booty		
S. Seftenberg	0		Genovar 2156 (1)	Scalder/picker inedible barrel
TOTAL MATCH	30/50 (60%)		20/50 (40%)	
Salmonella species confirmed	50/50 (100%)			

Table 13: Comparison of Kaufmann-White (KW) and PTS results of fresh isolates collected from PORK

Salmonella Serotype (KW)	Salmonella PTS Results			
	Complete Match	**Locations**	**Other I.D. (# Isolates)**	**Locations**
S. Anatum	14	Inedible cart, head, head without hide, feces, hide puller, ears, foot, hide conveyor, ground pork, offal	Genovar 16111 (5)	Offal, hide, foot, ears, head
S. Ohio	6	Inedible cart, offal, hide puller chain, foot, inedible conveyor belt, post evisceration conveyor	Genovar 16077 (1)	hide
			Unidentified (1)	foot
S. Typhimurium var. Copenhagen	0		Genovar 11935 (1)	Stomach
			Unidentified (1)	Chunk trim meat
S. Derby	1	tongue	Unidentified (1)	head
			Adelaide (1)	inedible
S. Heidelberg	3	Inedible cart, hide puller		
S. Mbdanka	2	Ears, head	Genovar 11949 (1)	Hide puller
S. Adelaide	1	Stomach		
S. Agona	0		Altona (1)	Head rack
S. Bovis-morbificans	0		Genovar 15607 (1)	offal
S. Manhattan	1	offal		
S. Newport	0		Unidentified (1)	Inside barrel
S. Saint Paul	0		Unidentified (1)	hide
S. Johannesburg	0		Urbana (1)	offal
S. Typhimurium	2	Ground meat, head		
Not Salmonella	2	Ground pork, feces		
Unknown by KW	1		Genovar 7540	Pen feces
TOTAL MATCH	33/50 (66%)		17/50 (34%)	
Salmonella species confirmed	50/50 (100%)			

II. Antimicrobial Resistance

A. *Sample collection*

Salmonella isolates were collected from seven pork and poultry plants throughout the year and in different states as indicated in Table 14. One hundred samples resulted in positive isolates out of 285 total samples collected; strains were coded to identify the specific place where they were collected within each plant. Each isolate was serotyped using the traditional Kauffman- White method and the Premi®Test *Salmonella* system (results discussed previously), subsequently these fresh isolates were subtyped using PFGE and their antibiotic resistance was studied as well.

Every possible source of *Salmonella* within the slaughtering process and its environment was sampled. The purpose was to obtain at least 100 isolates (50 from poultry and 50 form pork) that could represent as many *Salmonella* serotypes as possible. Samples were collected by a group of collaborators from Texas A&M, and assigned a code to designate whether they came from poultry (A) or pork (P) sources. Tables 15 and 16 contain detailed information about the origin of each sample. A majority of the positive isolates were obtained from inedible sources although the sampling included carcasses, knifes, cutting tables, and saws among other equipment and tools in direct contact with the product that could be potential sources of contamination.

Table 14: Summary of information of poultry and pork plants sampled

	Type	State	Season	Samples Collected	Positive Samples	Sample Code
Plant A	Poultry (ARS samples)	TX	Throughout the year	38	38	A1-A44
Plant B	Pork	TX	Set 1 - Sept./Oct. '09	16	2	P1, P2
			Set 2 – Nov. '09	38	2	P3,P4
Plant C	Pork	TX	Dec. '09	62	10	P12-24
Plant D	Pork	IL	Jan. '10	40	24	P5, P6, P25-46
Plant E	Pork	WI	Jan '10	35	10	P47-56
Plant F	Pork	IL	Jan '10	26	2	P57-58
Plant G	Poultry	TX	Jan '10	30	12	A51-65

Table 15: *Salmonella* isolates collected from poultry sources

Plant	Code	Source	Plant	Code	Source
Plant A	A1	Litter	Plant A	A26	Ceca
Plant A	A2	Soil Outside	Plant A	A27	Ceca
Plant A	A3	Soil Inside	Plant A	A28	Inside beetle
Plant A	A4	Water	Plant A	A29	Ceca
Plant A	A5	Litter	Plant A	A30	Litter
Plant A	A6	Water	Plant A	A31	Water
Plant A	A7	Feed	Plant A	A32	Inside beetle
Plant A	A8	Soil inside	Plant A	A33	Carcass rise feathers on
Plant A	A9	Booty	Plant A	A34	Carcass rise feathers on
Plant A	A10	Outside beetle	Plant A	A36	Booty
Plant A	A11	Inside beetle	Plant A	A40	Water
Plant A	A12	Feed	Plant A	A43	Inside beetle
Plant A	A13	Booty	Plant A	A44	Litter
Plant A	A14	Booty	Plant G	A51	Scalder
Plant A	A15	Soil Inside	Plant G	A52	Scalder
Plant A	A16	Litter	Plant G	A53	Scalder/picker inedible barrel
Plant A	A17	Inside beetle larva	Plant G	A54	Scalder/picker inedible barrel
Plant A	A18	Booty	Plant G	A55	Chicken feet
Plant A	A19	Soil Outside	Plant G	A56	Live chicken loader conveyor
Plant A	A20	Inside beetle	Plant G	A57	Live chicken loader conveyor
Plant A	A21	Ceca	Plant G	A58	Live chicken loader conveyor
Plant A	A22	Inside Beetle	Plant G	A59	Chicken after picking
Plant A	A23	Ceca	Plant G	A60	Chicken after picking
Plant A	A24	Beetle larvae	Plant G	A64	Feet chute
Plant A	A25	Outside beetle	Plant G	A65	Inedible barrel evisceration

Table 16: Pork isolates collected from swine sources

Plant	Code	Source	Plant	Code	Source
Plant B	P1	Stomach	Plant D	P34	Hide puller chain
Plant B	P2	Stomach	Plant D	P35	Ears
Plant B	P3	Inedible barrel	Plant D	P36	Ears
Plant B	P4	Head rack	Plant D	P37	Pen feces
Plant D	P5	Ground meat	Plant D	P38	Head
Plant D	P6	Chunk trim meat	Plant D	P39	Foot
Plant C	P12	Pig foot	Plant D	P40	Hide
Plant C	P13	Pig foot	Plant D	P41	Tongue
Plant C	P16	Inedible conveyor belt	Plant D	P42	Foot
Plant C	P17	Post evisceration conveyor	Plant D	P43	Offal
Plant C	P19	Hide	Plant D	P44	Head
Plant C	P20	Inedible cart	Plant D	P45	Head
Plant C	P21	Offal	Plant D	P46	Head w/o hide
Plant C	P22	Head	Plant E	P47	Hide conveyor
Plant C	P23	Hide puller chain	Plant E	P48	Ground pork
Plant C	P24	Ground pork	Plant E	P49	Inedible cart
Plant D	P25	Hide puller chain	Plant E	P50	Inedible cart
Plant D	P26	Offal	Plant E	P51	Inedible
Plant D	P27	Offal	Plant E	P52	Offal
Plant D	P28	Inedible barrel	Plant E	P53	Inedible
Plant D	P29	Head	Plant E	P54	Hide puller
Plant D	P30	Hide	Plant E	P55	Offal table
Plant D	P31	Foot	Plant E	P56	Head
Plant D	P32	Pen feces	Plant F	P57	Offal
Plant D	P33	Pen feces	Plant F	P58	Feces

B. *Antibiotic Resistance*

Among 100 *Salmonella* fresh isolates tested against a panel of six antimicrobials, it was found that 14 isolates were resistant to at least one of the antimicrobials evaluated. The antibiotic resistant isolates and their antibiotic patterns are summarized in Table 17. Resistance to an antimicrobial was determined by comparing the values read by the Flash & Go Automatic counter with the values recommended by the Hardy Disks'

manufacturer (Table 7). Strains were classified as resistant if they grew at the following antibiotic concentrations ampicillin (10 µg), chloramphenicol (30 µg), nalixidic acid (30 µg), streptomycin (10 µg), tetracycline (30 µg) and sulfixozasole (250 µg).

Tetracycline was the antibiotic which most strains were resistant, within this group of resistant strains *S*. Anatum (5 isolates), *S*. Heidelberg (3 isolates), S. Typhimurium (var Copenhagen) (2 strains), *S*. Newport (1), *S*. Mbandaka (1), *S*. Bovis-morbificans (1), and *S*. Urbana (1) were represented. All of these isolates were collected from pork sources. Multidrug resistance was observed in both *S*. Typhimurium isolates and in S. Bovis-morbificans, which were resistant to G-AM-C and Te-G-AM respectively. Resistance to nalidixic acid and streptomycin was not observed in any of the isolates, independently of the host animal or the site of collection.

Table 17: Antibiotic resistant strains and patterns. Abbreviations are as follows: Te = tetracycline, C= chloramphenicol, G= sulfixozasole , Am = ampicillin.

Serotype KW	Host	Source	Resistance Pattern
S. Anatum (5)	Pork	Offal, head, pen feces	Te
S. Bovis-morbificans (1)	Pork	Offal	Te-G-Am
S. Heidelberg (3)	Pork	Inedible cart	Te
S. Mbandaka (1)	Pork	Ears	Te
S. Newport (1)	Pork	Inside barrel	Te
S. Typhimurium (var Copenhagen) (2)	Pork	Stomach/Chunk trim meat	G-AM-C
S. Johannesburg (1)	Pork	Offal	Te

Plant D contributed to 59% of the Tetracycline resistant strains. *S.* Anatum was the most frequently isolated serotype in this plant and two genotypes (G4 and G5) were identified. Plant E, located in Wisconsin, represented 33% of the strains resistant to this

antibiotic followed by plant B, located in Texas, which contributed with 8%. Only one genotype was identified for each *Salmonella* serotype collected from these two plants (*S*. Heidelberg, *S*. Bovis-morbificans, and *S*. Newport). Despite the fact that most samples were collected from plant C neither of these isolates showed resistance to any of the antibiotics tested nor the ones collected from plant F.

III. Pulse Field Gel Electrophoresis

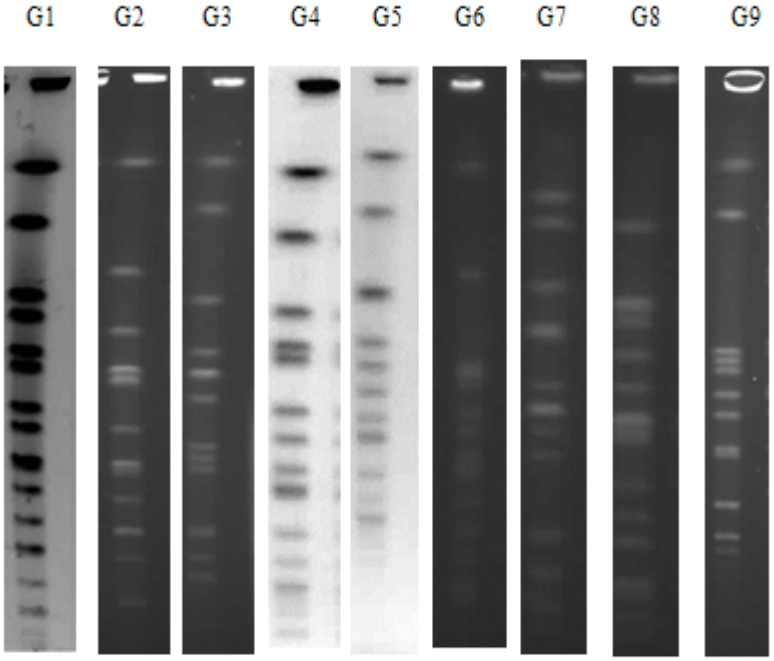
A. *PFGE macrorestriction profiles*

Salmonella isolates collected from pork and poultry sources were further analyzed using PFGE following the Pulse- Net CDC protocol. The PFGE patterns were generated by using the enzyme *XbaI*, which cuts 5´…T˅CTAGA…3´ sequences. Seventeen different serotypes were represented in the tested isolates, while the results from the PFGE analysis yielded 18 patterns designated G1 though G18 (Figures 3 and 4). Four serotypes had two PFGE genotype profiles and three other could not be restricted. A total of 20 isolates belonging to *S*. Kentucky (11 isolates), *S*. Ohio (8 isolates) and *S*. Saint Paul (1 isolate) were considered "untypable" because distinguishable bands were not generated in these strains by *XbaI* enzyme. These isolates were streaked on selective media and biochemical tests were conducted on all "untypable" strains to verify the purity of the culture, and to confirm that they were *Salmonella.* Also, a shorter restriction period was used in an attempt to obtain detectable fragments, but this was unsuccessful in solving the problem.

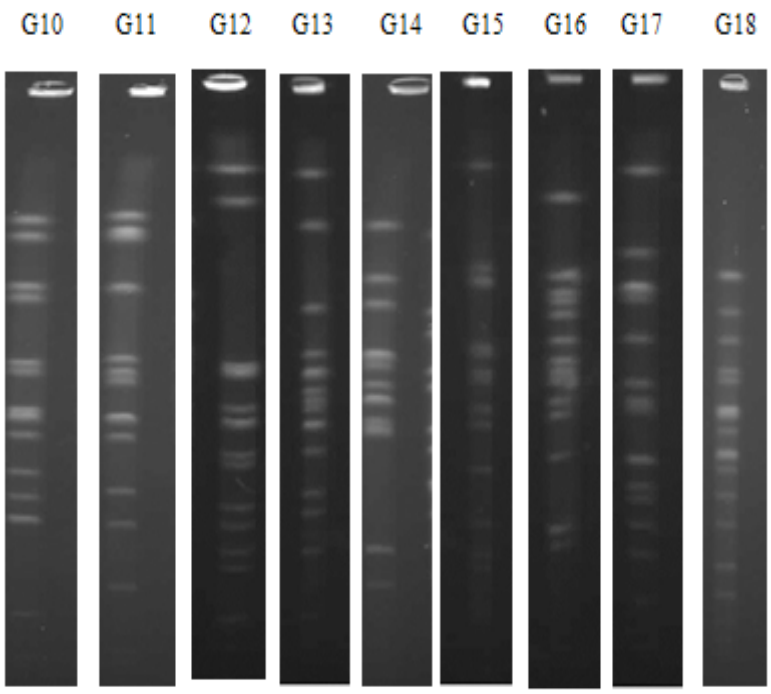
Salmonella serotype Braenderup (H9812) restricted with *XbaI* is the standard strain used by Pulse Net because of its even distribution of bands over the entire range of band sizes

normally seen in foodborne pathogens. Fragment sizes from this strain are provided in the literature and were used to determine the bands' size in the PFGE patterns found among the fresh isolates. Most of the genotypes generated 11 fragments but the first nine were the clearest bands to detect therefore those were used for the determination of their molecular weight, which ranged from ~ 55kb to ~1058 kb. Figures 3 and 4 contain the PFGE patterns found in the fresh isolates from pork and poultry sources and the representations with their molecular sizes

Figure 3. PFGE patterns from Xabl restriction



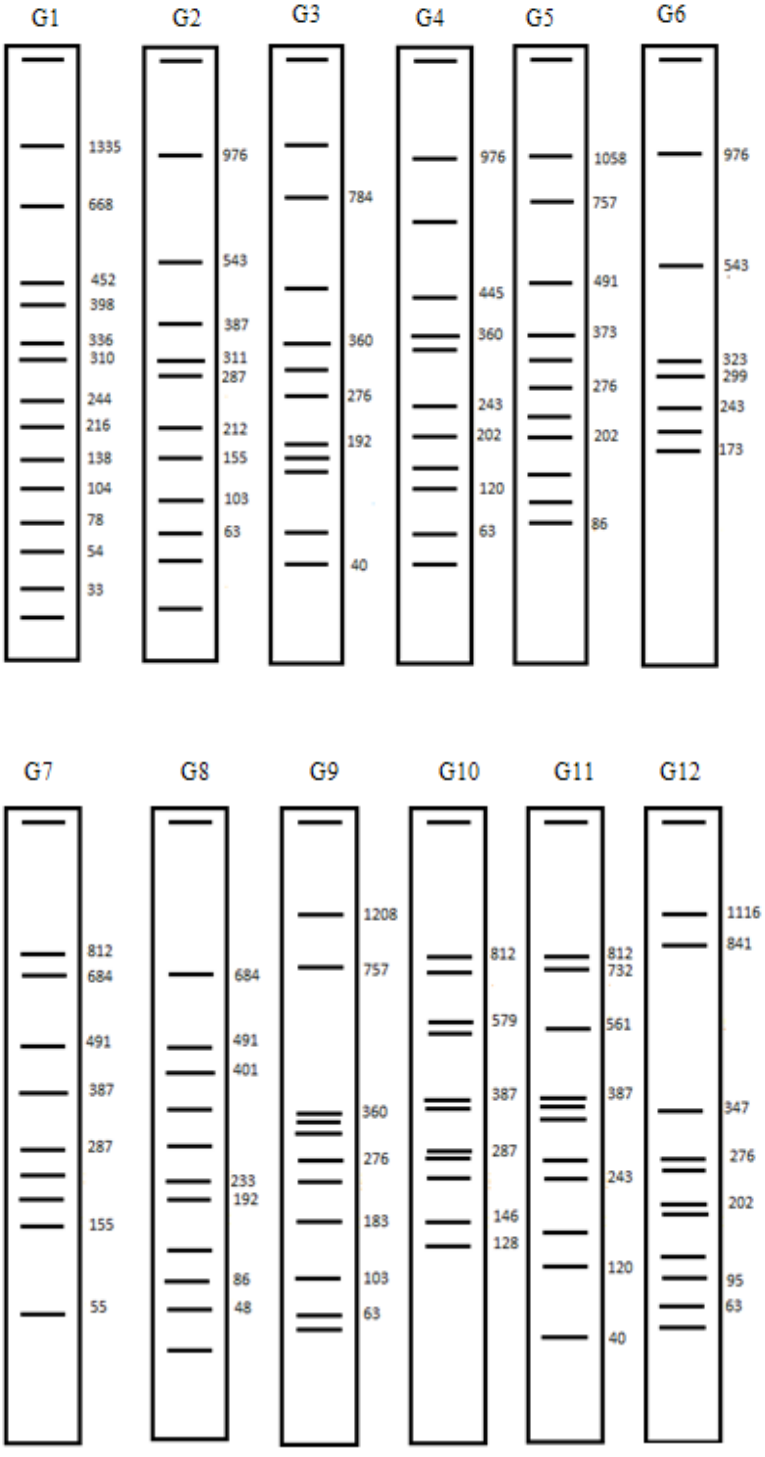
G1: *S.*Braenderup
G2: *S.*Newport
G3: *S.*Senftenberg
G4: *S.*Anatum
G5: *S.*Anatum *
G6: *S.*Braenderup *
G7: *S.*Typhimurium
G8: *S.* Heidelberg
G9: *S.*Bovis-morbificans

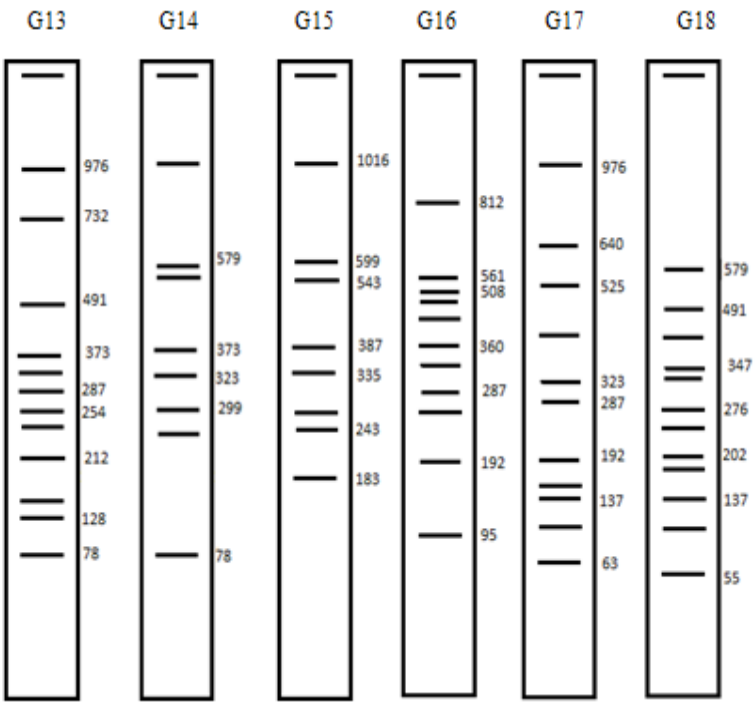


G10: *S.*Adelaide
G11: *S.* Derby
G12: *S.*Newport*
G13: *S.*Derby*
G14: *S.*Agona
G15: *S.*Manhattan
G16: *S.*Johannesburg
G17: *S.*Mbandaka
G18: Unknown (Genovar 7540)

* Serotypes with more than one PFGE pattern

Figure 4: Schematic representation of the PFGE patterns with molecular weights alone the side





B. ***Dendrogram***

A dendrogram of the PFGE patterns generated by using *Xbal* was created using the MarkFind software. Bands obtained for each macrorestriction profile were compared and scored in a spread sheet using a binary code (1 if the band was present or 0 if it was not). The spread sheet was saved in a cvs format and then imported to the MarkFind program, which uses the UPGMA (Unweighted Pair Grouping Method with Arithmetic-mean); which is one of the simplest and most commonly used hierarchical clustering algorithms. The dendrogram generated 10 clusters (Figure 5), two from poultry and eight from pork. Isolates from the same host (poultry or pork) and collected from the same plant were clustered together indicating the high specificity of the serotypes to a specific host and environment (figure 6 and figure 7).

Figure 5. Dendrogram of *Salmonella* isolates. Poultry (A) and pork (P) isolates are showed. Numbers located at the node indicates the percentage of relatedness.

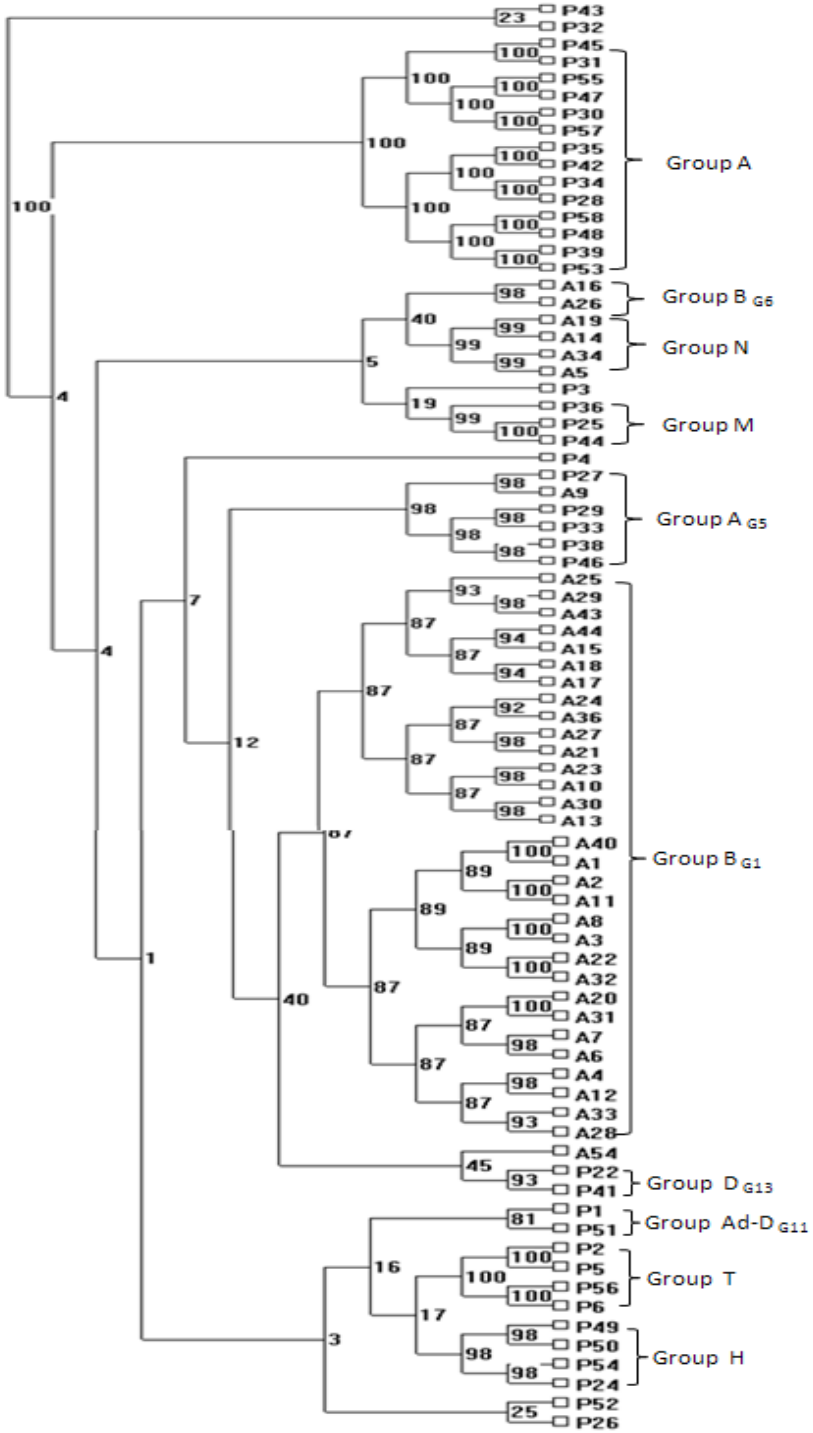


Figure 6. Dendrogram of *Salmonella* isolates from poultry sources. Plants of collection and serotypes are noted on the right site. Numbers located at the node indicates the percentage of relatedness.

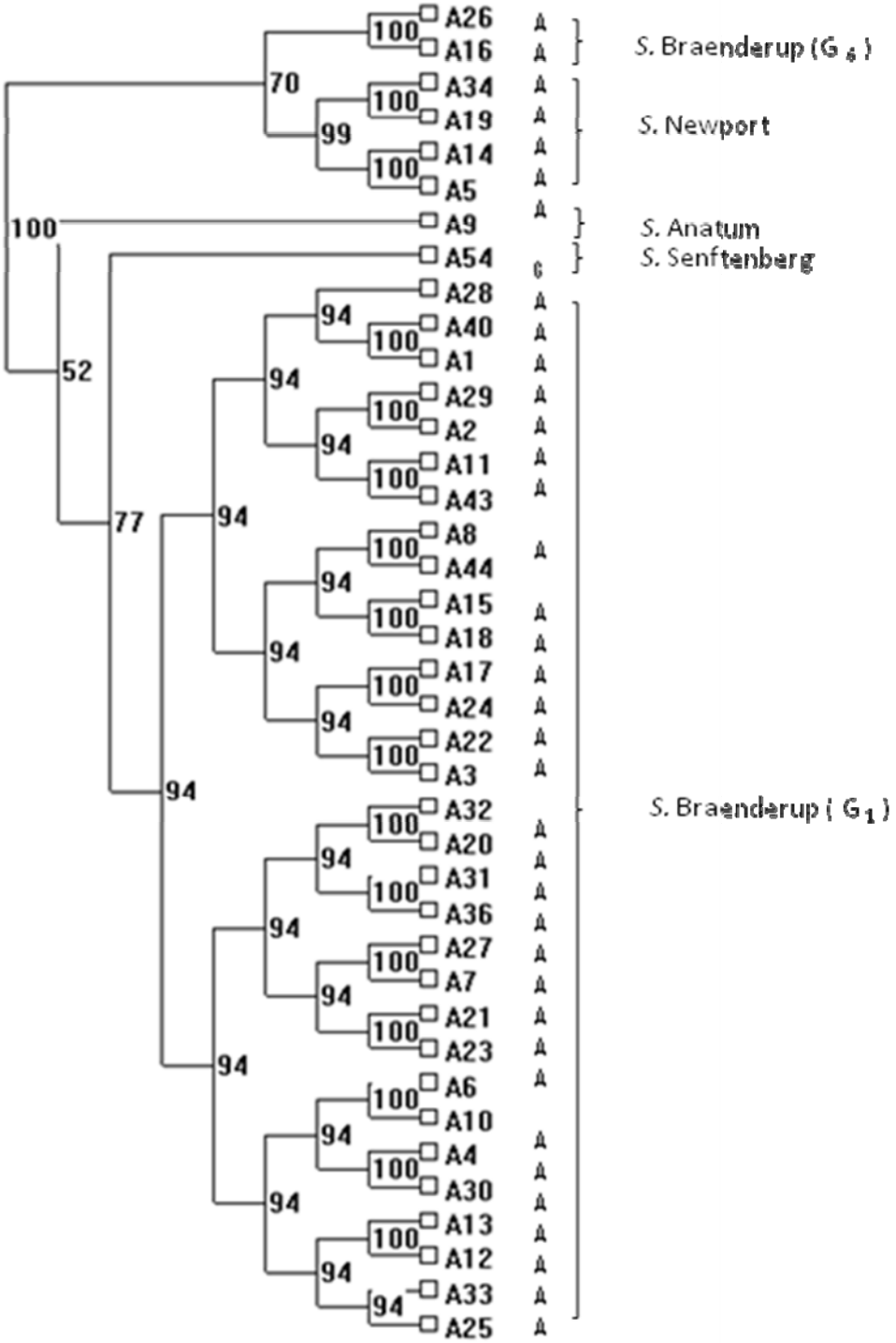
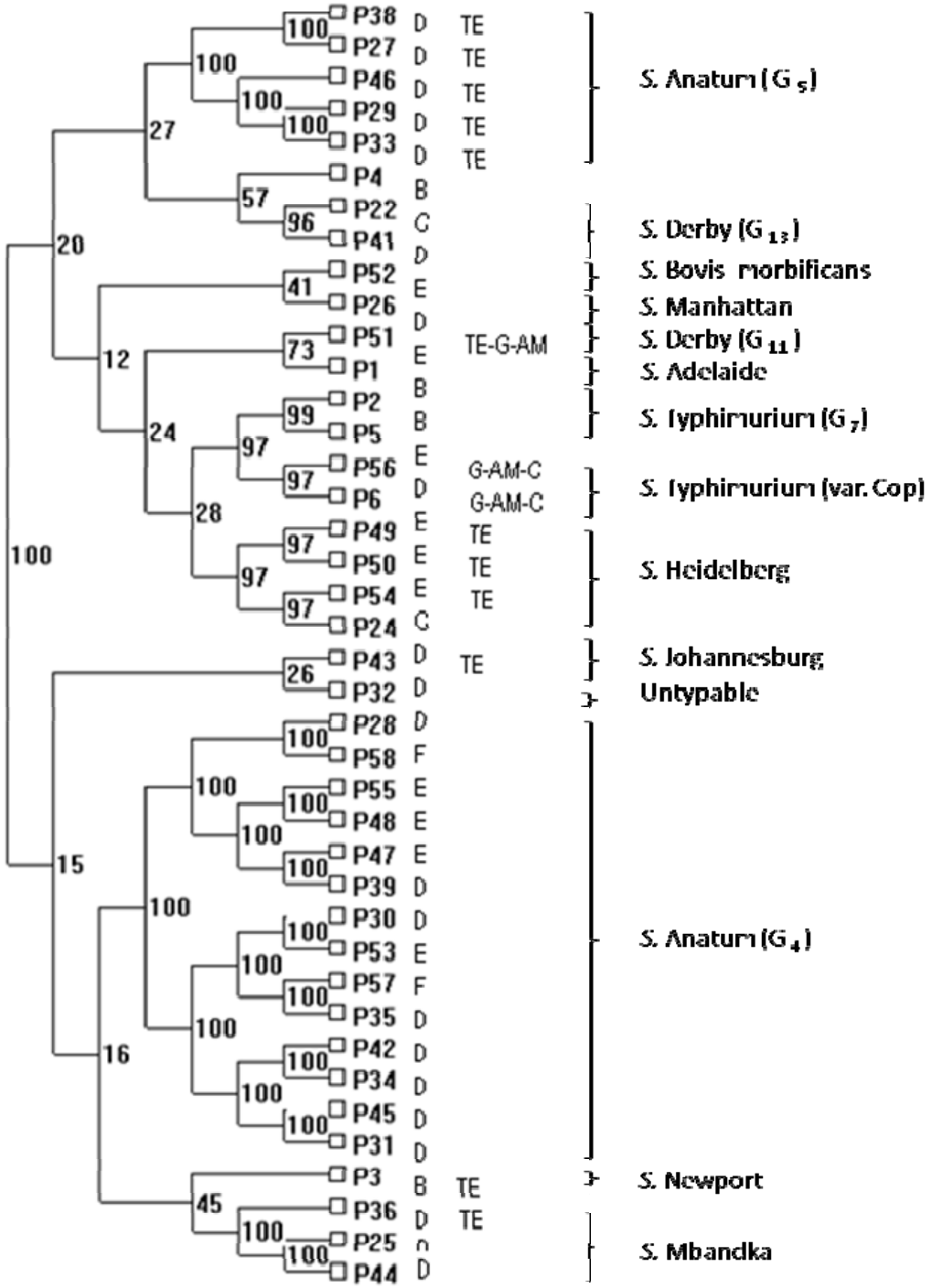


Figure 7. Dendrogram of *Salmonella* isolates from poultry sources. Plants of collection, antimicrobial patterns, and serotypes are noted on the right site. Numbers located at the node indicates the percentage of relatedness.



B. *Environmental sources, potential reservoirs for Salmonella*

Results on the prevalence of *Salmonella* serotypes depends on method of isolation that could be favorable for the recovery of some serotypes (49). The prevalence of other species differs widely both spatially and temporally. Therefore the results from this study should be confirmed with further investigations.

Infected birds, contaminated feed or infected rodents normally result in horizontal contamination (47). Thirty three percent of the total number of isolates from poultry sources were collected from booty, litter, water and feed (Table 18). Soil was an important source for *Salmonella* positive isolates and consequently beetles inside and outside the houses which contributed with 40% of contaminated samples.

Table 18: Sites of *Salmonella* collection from poultry sources. A= *S.* Anatum, B= *S.* Braenderup, K= *S.* Kentucky, N= *S.* Newport, S= *S.* Senftenberg.

Source	Total Positives	Serotypes
Booty	7	A=1,B=3,K,2,N=1
Litter	5	B= 4, N= 1
Soil outside	2	B=1, N=1
Water	4	B= 4
Feed	2	B= 2
Soil Inside	3	B= 3
Outside beetle	2	B=2
Inside beetle	8	B=8
Ceca	5	B=5
Carcass rinse feathers on	2	B= 1, N= 1,
Scalder	4	K= 3, S= 1
Live chicken loader conveyor	3	K= 3
Chicken after picking	2	K= 2
Inedible barrel	1	K= 1

The equipment used during the slaughtering process was identified as an important source of cross contamination in isolates collected from swine sources. Hide puller and

hide represented 20% of the positive samples (Table 19). In addition, some by products (head, foot and ears) that could be sold or transformed into another product and reach the consumer are sources of special concern, as well as the occurrence of positive *Salmonella* on ground meat which together accounted for 30% of the positive isolates collected in swine plants.

Table 19: Sites of *Salmonella* collection from swine sources. A= *S.* Anatum, AD= *S.* Adelaide, AG = S. Agona, BM= *S*. Bovis-morbificans, D= *S*. Derby, H= *S*. Heidelberg, J= *S*.Johannesburg, M= *S*. Manhattan, MB= *S*. Mbandaka N= *S.* Newport, O= *S.* Ohio, SP= *S.* Saint Paul, T= *S.* Typhimurium, TC= *S.* Typhimurium (var. Copenhagen), U= Untypable (Genovar 7540)

Sources	Total Positives	Serotypes
Pen feces	4	A= 3, U= 1
Offal	7	A= 3, BM= 1, J= 1, M= 1, O= 1
Inedible barrel	7	A= 2, D= 1, H= 2, N= 1, O= 1
Head	8	AG= 1, A= 4, D= 1, MB= 1, T= 1
Hide	3	A= 2, SP= 1
Foot	5	A= 3, O= 2
Hide Puller	7	A= 1, H= 1, MB=1, O= 4
Ears	2	A= 1, MB= 1
Tongue	1	D= 1
Stomach	3	AD= 1, T= 1 , TC= 1
Ground meat	3	A= 1, T= 1, TC= 1

CHAPTER IV

DISCUSSION

I. Premi®Test system vs. Traditional Kauffmann- White Method

Overall, in tests with the USDA culture collection, the PTS results appeared to be reproducible independent of the source (pork or chicken). Sixty three percent of the serotypes present in the PTS database matched traditional serotyping, and all isolates were identified as *Salmonella*. Thirty seven percent of the isolates present in the database were identified as *Salmonella* but did not match results from traditional serotyping. Further investigation may lead to discrepancies due to mistyping of the original isolates by the traditional method or overlaps with known serotypes. It has also been observed that serotypes can change over time depending on a number of factors such as storage and growth conditions (USDA, personal communication). Certain isolates not present in the PTS database were recognized as *Salmonella* Genovars, although the profile was unknown. It was difficult to decide whether these should be declared a "match" or not because the inherent limitations of the database preclude making this determination. Although some serotypes were not present in the database, the system did correctly identify these isolates as *Salmonella* species 96% of the time, indicating that the generic microarray markers were very accurate in determining species.

Among the 66 poultry isolates from the USDA collection, 56 different serotypes were represented; 27 isolates were not present in the PTS data base (Table 8) and 39 were present (Table 9). Thirty four USDA isolates from pork were evaluated, and 30 different serotypes were represented in this group. Serotypes which were not present in the PTS database are shown in Table 10. Most were assigned a different serotype from ones present in the database, except for *S*. Uganda which yielded a Genovar score. A

majority of the serotypes present in the PTS database (Table 11) matched the serotyping results from the traditional method.

Serotyping of the fresh isolates yielded some interesting data. Both methods correctly identified the species as *Salmonella* 100% of the time. Two serotypes, *S*. Kentucky and *S.* Braenderup comprised 88 % of the total number of isolates found in chicken; while *S.* Ohio and *S.* Anatum made up 54% of the isolates found in pork (Tables 12 and 13). Among the fresh isolates only one serotype, *S*. Johannesburg from pork, was not included in the PTS database. Thirty out of fifty isolates from poultry fully matched with KW results which represent 60% of the total number of isolates from poultry; the remaining 40% yielded a Genovar score, a different serotype, or the report that the identification of the serotype was not possible (Table 12). However, the system was not able to identify *S*. Kentucky, although this serotype is claimed to be part of the database. Sixty six percent of the isolates from pork matched the results from the traditional method (Table 13).

A total of 200 isolates were evaluated using both the traditional Kauffman – White method and the Premi®Test *Salmonella* system. From the USDA isolates a wide variety of serotypes from poultry and pork were assessed, with 49% and 76% of successful matches respectively, between the two methods occurring when the isolates were present in the database, if not, a Genovar score was generated. The presence of the genetic markers of the genus *Salmonella* were detected 100% of the time. The results from serotypes present in the PTS data base that did not match the traditional method could be explained by a possible overlap with the profiles of those serotypes present in the database due to a close evolutionary relationship. It is also possible that *Salmonella*

serotypes isolated in the United States have enough antigenic differences from their European counterparts to cause mismatches within the microarray, which was produced, manufactured, and validated in the Netherlands. According to a surveillance conducted on the world-wide distribution of *Salmonella* from 2000-2002, *S*. Enteritidis accounted for 85% of *Salmonella* cases, whereas *S*. Typhimurium was the most common human isolate (29%) in North America. In addition, the variety of serotypes in the U.S. was more evenly distributed with *S*. Enteritidis (21%), *S*. Newport (15%), and *S*. Heidelberg (10%) accounting for a sizeable proportion of the isolates (68).

S. Ohio and *S*. Anatum were the most frequently found serotypes isolated from pork sources, which represented over 50 % of the total number of fresh samples collected form pork processing plants. Similar results were observed by Ross *et al*.(70). They reported on the prevalence of *Salmonella* in environmental farm samples and found *S.* Anatum to be the most commonly isolated serovar at 48.4% from 2,496 farm samples. The USDA reported that the five most frequently isolated *Salmonella* serotypes from swine collected from 1998 to 2000 were *S*. Derby, *S*.Typhimurium var. Copenhagen, *S.* Johannesburg, *S.* Infantis and *S.* Heidelberg (78). None of the most frequently isolated serotypes in this study fell into this group. Another interesting observation is that *S.* Ohio and *S*. Anatum are not listed among the top 20 most commonly reported serotypes from human sources (59)

The results from this study indicate that although swine and poultry environments are reservoirs for *Salmonella*, the serotypes frequently reported in the literature to be most prevalent may not be representative of all plants and all regions of the United States. Much larger studies are needed to corroborate these findings.

S. Kentucky and *S.* Braenderup represented 88% of the fresh samples isolated from poultry sources in this project. *S.* Braenderup is the 12th most often isolated serovar from human sources while *S.* Kentucky is not even listed as a human isolate. *S.* Kentucky appears to be the most prevalent *Salmonella* serovar in chicken (63,75).

Although this serovar does not cause invasive disease, some isolates have been shown to possess the MDR-AmpC multidrug resistance pattern (75). It is important to consider the possibility of other *Salmonella* serotypes acquiring resistance genes from *S.* Kentucky (75). Larger studies and increased sampling will help to determine if the number of resistant strains is increasing in poultry processing plants around the country.

II. Antibiotic resistance

Salmonella has become the focus of discussion on the global dissemination of antimicrobial resistance genes (56, 65). Results from *Salmonella* resistance surveillance is used as an indicator of the status of resistance in other zoonotic pathogens (56).

A total of 100 isolates were collected from poultry and swine sources from different points along the process and the environment. These isolates were tested for susceptibility to 6 antimicrobial agents of human health significance. Interesting, antibiotic resistance among poultry isolates was not observed although 88% of the isolates from poultry sources were *S.* Kentucky and *S.* Braenderup, two serotypes that have been reported to be resistant to at least two antibiotics tested in this study (tetracycline and ampicillin) (42)**.** Seventy two percent of the isolates from pork did not show resistance to any of the antimicrobials evaluated.

All *Salmonella* serotypes are considered pathogenic and therefore represent a hazard for public health (9). However, the rate of resistance to antibiotic agents varies among serotypes (9). In this study, high resistance to tetracycline was observed on swine isolates independently of the serotype (Table 17) or the plant (Table 14) where they were collected, indicating that resistance to this antibiotic agent is spread in the United States. Tetracycline is commonly used in animal feed to promote growth and resistance to this drug has commonly been observed (63, 67).

S. Anatum, *S*. Heidelberg, S. Mbandaka, *S*. Newport and *S*. Johannesbug all showed resistance to tetracycline, which has been reported in other studies (61,65).

Two multidrug resistance patterns were detected. G-AM-C (sulfixozasole, ampicillin and chloramphenicol) pattern was observed in *S*. Typhimurium (var. Copenhagen) even though, they came from different plants (plant B and plant D) located in different states (TX and IL), and were collected in different months of the year (Nov. 09 and Jan. 10). In addition, the TE-G-AM (tetracycline, sulfixozasole and ampicillin) resistance pattern was observed in *S*. Bovis-morbificans.

S. Typhimurium, including variant Copenhagen, have often been found to be resistant to more than five antibiotics including ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracyclines (9,56,65). These findings are consistent with the results in this study where 100% of the *S*. Typhimurium var. Copenhagen showed a multidrug resistant pattern G-AM-C.

Gene encoding for tetracycline resistance and multidrug resistance in gram negative bacteria have been found associated with mobile genetic elements that encode specific

resistance genes (71). Therefore it is of great importance to monitor antibiotic resistance among *Salmonella* isolates from poultry and pork sources to detect emerging resistant pathogens and antibiotic resistance trends.

III. Pulsed Field Gel Electrophoresis

Previously it was discussed that sixty three percent of the serotypes present in the PTS database matched traditional serotyping and thirty seven percent of the isolates present in the database did not match results from traditional serotyping. The PTS system was able to correctly identify all isolates as *Salmonella* species but when mismatches occur with results from the KW scheme the system reported either a genovar score or alternative serotype. The PFGE profiles helped in the discrimination of mismatches encountered between traditional serotyping with the Kauffman-White method and the Premi ®Test system. In most of the cases (83%), when the PTS system reported an alternative serotype, the PFGE pattern confirmed the serotype found with the KW scheme. This findings show a higher discriminatory power of the KW method over the current PTS system.

Minimum genetic variation was observed. PFGE profiles remain stable among isolates belonging to the same serotype. Two genotypes were observed from *S*. Derby, *S*. Braenderup, and *S*. Anatum (Figure 3). PFGE studies indicate that mismatches from the PTS system are not due to the close relatedness among isolates. Each cluster presented a different scenario in terms of the results reported with the PTS system in comparison with the KW method. Therefore each group will be analyzed as an individual case as follows.

Isolates form *S*. Anatum were grouped in two clusters (Group A and Group to A_{G5}). Genovar 16111 was reported for seven isolates but they were present in both clusters even though, the groups were not close related to each other (Figure 5). The system has the potential to identify both genotypes (5 and 6) but there was not consistency on the reported results.

As in the previous case *S*. Braenderup formed two groups (B_{G1} and B_{G6}). The PTS system was not able to identify the isolates with the G6 pattern and provided the genovar score 9614. Nevertheless this genovar score was not assigned to any other isolate that could not be identified by the system. These findings indicate that the PTS system is able to distinguish between this two genotypes but microarray profile has not been found frequently enough to integrate it to the database.

S. Typhimurium var. Copenhagen could not be recognized by the PTS, but the system did not report it as *S*. Typhimurium either. Thus, the system has the discriminatory power to detect the differences between these strains even though they are highly related in the phylogenetic tree.

S. Heidelberg and *S*. Mbandaka had 100% phylogenetic relatedness among isolates within each group (group H and group M respectively). Successful matches were reported with the PTS for all these isolates.

S. Derby had two genotypes (G11 and G13). Low relatedness was observed between these two genotypes (<50%). The PTS system reported one isolate from *S*. Derby (G11) as *S*. Adelaide (G10). The overlap was produced because of G10 and G11 are closely related (81%).Two bands (size 491 kb and 146 kb respectively) absent in *S*. Derby

(Figure 4) may have allowed the system generate an erroneous result . This was the only case when the hypothesis of overlaps due to close related strains was confirmed.

In summary, when isolates generating a genovar score did not successfully match other isolates within the same cluster, the reason for the mismatch was most likely due to darker or weaker spots generated in the microarray profile; which did not allow the system to associate the resulting profile with the one in the data base.

IV. Epidemiology

Previous studies have demonstrated the food safety significance of various environmental sources as potential contributors to *Salmonella* dissemination from the farm throughout the slaughtering process (26). *Salmonella* control and reduction interventions can be implemented at three levels with the food production chain: pre-harvest (on farm), harvest (transport and slaughter house) and post-harvest (cutting, processing, retail and preparation at home).

Rodriguez et al. investigated the distribution of *Salmonella* in a broader scenario involving a variety of farm types (poultry, beef, cattle, dairy, and swine) (68). They found that feed stuff, soil, bedding, litter and feces were notable sources of *Salmonella*. Specifically, the prevalence in poultry farms and swine was 3% and 10.7 % respectively (68). According to the data showed in Table 18 samples collected from booty, litter, water and feed accounted for 31% of the total positive samples recovered from poultry sources. These sources have been identified as important contributors for horizontal contamination. Soil can be contaminated with animal faces, wild birds, water irrigation, rodents (68), consequently the contamination can be spread to chickens and from them

to the water and feed, as well as to insects which become potential carriers of *Salmonella*. Beetles found inside and outside of the house tested positive for *Salmonella* representing 40% of the positive isolates. *Salmonella* and other human pathogens can be carried by insects in their legs and bodies this can result in human diseases, such as food poisoning or diarrhea (36,39,76). Occurrence of horizontal contamination was confirmed with the findings in this study . Isolates form *S.* Braenderup and *S.* Newport were detected on several sites of the process from farm to plant samples (Table 18).

Several routes of transmission (feed, equipment, facilities, personnel) have been studied to explain the epidemiology of *Salmonella* in pigs (50). Table 19 indicates that 20% of the positive isolates were recovered from the equipment used for deharing and mobilization of the carcasses through the slaughtering process (hide puller and hide). *S.* Anatum (Genotype 4) was isolated from hide and hide puller and also from ground beef, which indicated cross contamination produced by these devices.

Thirty percent of the samples collected from heads, feet, ears and meat tested positive for *Salmonella*. These products could be sold or further processed and transformed into pet food become hazardous for the consumer's health. The fact that 43% (6 out 14) of the antibiotic resistant isolates came from these sources increases the risk of infection due to consumption of any of these products.

S. Anatum and *S.* Typhimurium are frequently isolated from humans and commonly associated with food borne illnesses (15, 43 , 78). According to the findings in this study these serotypes are capable of surviving the interventions throughout the process. *S.*

Typhimurium and especially *S.* Anatum were isolates detected in earlier steps of the process and also in the final product (ground meat).

CHAPTER V

CONCLUSIONS

The Kaufmann-White traditional serotyping method based on antibody-antigen reactions is considered the gold standard for typing *Salmonella* species. However, the method does possess deficiencies in that it is time-consuming, results are sometimes not reproducible, highly experienced personnel are required to perform the test, and the availability of sera can be limiting. The PTS system is a DNA based method which targets genetic information of different serovars for the purpose of identifying the serotype in addition to the genus *Salmonella*. The PTS system's processing time of 8-9 hours after enrichment and isolation is highly attractive for high-throughput laboratories. The PTS system is relatively simple to use and previous research has indicated a high specificity for the 100 serotypes present in the data base (79). The procedures are standardized and therefore should be more easily reproducible from laboratory to laboratory. The use of this system has the potential of increasing the accuracy of serotyping and decreasing the time to result of analysis, which are important factors when responding to outbreaks or when monitoring sanitary controls in flocks or slaughter operations. Although complete differentiation between all serotypes is not yet possible in this system, future releases of the PTS software should include new identifiers that will expand the database.

The PTS system has tremendous potential for additional growth, expansion, and research even though the results of this study indicate that it does not yet possess the discriminatory power necessary to replace traditional serotyping. It is recommended that companies and research institutions interested in this technology maintain links to traditional serotyping methodologies to verify the instrument and work hand-in-hand with the manufacturer to identify difficult serotypes. Genovar scores should be analyzed

in greater depth to find if they correspond to unique serotypes in different locations. Also, specific practical and technological issues need to be addressed. For example, the price of the kits is quite high ($3,200 for 72 samples), and the incubation time for the final detection step seemed to be inconsistent from sample to sample. Although 15 minutes incubation is recommended, sometimes the reader would not produce any result or a correct result until a longer incubation was used. Sometimes the reader would report one serotype, and then a few minutes later would report a different one when it was read a second time. Correcting this issue will help avoid discrepancies in the future. The system also had trouble in identifying *S*. Kentucky, a common serotype in poultry in the U.S. There seems to be some evidence that the use of pure DNA extracts is better than crude extracts in increasing the accuracy of the device, therefore this may lead to more reliable results.

As the system and method evolves, it should continue to undergo rigorous testing on as many isolates from as many sources as possible that originate from all parts of the world. If perfected, this new technology could provide a means of rapid surveillance of *Salmonella* serotypes in the food chain and in epidemiological investigations.

Genetic relatedness is not the most important factor responsible for the discrepancy in results obtained with the Premi®Test system. The same genovar score was assigned for unsuccessful matches from isolates from different clusters which were not genetically closely related. Although the system was inconsistent, it was able to recognize both genotypes from *S.* Anatum; in some cases it correctly assigned the serotype to the sample matching with the traditional KW method, and for other isolates, from the same genotype, a genovar score was reported. Mismatches of *S.* Braenderup (genotype 6)

were correctly assigned a unique genovar score and clustered separately from genotype 1 isolates. Only once was it observed that close relatedness (81%) between *S*. Derby (genotype 10) and *S*. Adelaide produced an overlap on the results, reporting *S*. Derby as *S*. Adelaide .

PTS mismatches with the KW method could be occurring because of the absence of appropriate markers for the identification of endemic *Salmonella* strains found in poultry and plant sources in the United States. In addition, it is also recommended that the staining step in the Premi®Test procedure be improved. It is time sensitive and the formation of darker or weaker spots contribute to the erroneous interpretation of the microarray profile.

Pork sources are reservoirs for antibiotic resistant *Salmonella*. Resistance to tetracycline was detected in *S*. Anatum *S*. Heidelberg, S. Typhimurium (var. Copenhagen), *S*. Newport, *S*. Mbandaka , *S*. Bovis-morbificans , and *S*. Urbana. Multidrug resistance was detected on *S*. Typhimurium isolates and in *S*. Bovis-morbificans, which were resistant to G-AM-C and Te-G-AM respectively. Tetracycline resistant isolates were mainly collected from inedible sources nevertheless; they could be sources for horizontal contamination. *S*. Typhimurium multidrug resistant strains were isolated from ground becoming of higher risk for human health. A feature of multiresistant strains is the reduction of effectiveness of antibiotic in medical treatment (15). The use of chloramphenicol is banned in food- producing animals the United States because of its tendency to cause blood dyscrasia (34). It is indicative of the importance of monitoring systems to detect emerging antibiotic resistance trends.

Isolates were collected from different plants located in the United States. Predominant serotypes were specific for poultry (*S.* Braenderup) and for pork (*S.* Anatum). When isolates from different host animals were integrated into the same dendrogram they were clustered together into the same group indicating the strong genetic relationship among *Salmonella* strains from different plants in United States. When strains were analyzed by animal origin the isolates collected in the same plant were clustered in the same group indicating high specificity of the *Salmonella* strain to their environment. The reason for prevalence of some serotypes could be due to physiology and resistance to environmental conditions. In addition, isolation methods could favor the recovery of certain serotypes.

Horizontal contamination was detected in poultry and pork processing chain. As demonstrated in other investigations (54). In this study feed, water, soil, litter, insect, rodents were found to be reservoirs and vectors for contamination in poultry sources. The prevalence of some serotypes (i.e *S.* Anatum) in pork processing (starting from positive isolates from the animal itself, continuing in the equipment used for transportation and finally reaching the end product) indicates the strong tolerance of some strains to the reduction interventions currently applied in this process. It would be useful to study these most prevalent isolates when conducting further investigations for more suitable interventions targeting this pathogen. However this would not decrease the importance and the need of monitoring programs to detect other *Salmonella* serotypes taking over that niche.

In summary, the final conclusions from this study are:

1. The PTS does not yet possess the discriminatory power necessary to replace traditional serotyping. However, this system has tremendous potential for additional growth, expansion, and research.
    - Specific genetic markers may need to be incorporated in the kit in order to be capable of detecting other common *Salmonella* serotypes in the United States and genetic variations within the same serotypes currently present in the data base.
    - The final staining step needs to be improved in order to generate consistent profiles in the microarray and consequently a consistent result.

2. Subtherapeutic doses of commonly available antibiotics are still being used in pork production. *Salmonella* antimicrobial resistance remains as a major health concern.

3. Poultry and pork environmental sources, equipment and by products are reservoirs and vectors for *Salmonella* contamination.
    - *Salmonella* serotypes are widely spread in plants from the United States. Monitoring the most frequently isolated and highly prevalent *Salmonella* serotypes would provide a better evaluation of more suitable in-plant controls and in–plant interventions.

CHAPTER VI

RISK ASSESSMENT:

MODELING AND AVENUES FOR FUTURE RESEARCH

I. Introduction

The most effective and least expensive approach in preventing foodborne outbreaks due to consumption of contaminated end-products in the implementation of hazard analysis systems (7). HACCP plans have addressed hazard analyses in an informal manner but it is evident that determining both the likelihood of occurrence of a hazard and the severity of the consequences is necessary to provide an objective tool to identify hazards that require more control (7).

Risk assessment is an important decision-making process that can be used to establish adequate national regulations for food producers and processors. The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) have made important contributions in the development of risk assessment models for the management of public health hazards in food (28).

Results from this project were never intended to be used in the development of a template for the risk assessment of *Salmonella* prevalence in small pork and poultry industries. The collection of *Salmonella* isolates was done by other institution without a sampling program. The project began with one specific objective, which was the validation of the Premi®Test system, and the lack of the total number of samples collected from each specific source within the plant did not allow the estimation of the prevalence. In addition, detailed information of the interventions applied in each one of the plants was not available either.

However to make a contribution for future research in this area, an overview of risk assessment using a probabilistic risk modeling tool is provided.

A. Risk assessment components

Hazard Identification

Salmonella populations in the United States from poultry and pork sources are diverse, but some serotypes are more common in these environments. For example, *S*. Enteritidis is associated with poultry products (22, 28) while *S*. Typhimurium, *S*. Derby, *S*. Brandenburg and *S*. London are associated with swine (25,26). *S*. Heidelberg is ranked fourth as the most common serovars isolated from human sources and first among *Salmonella* serovars from non-clinical nonhuman sources in a 2007 report prepared by the CDC (67).

As previously discussed *Salmonella* serotypes causing human illness are frequently isolated from swine and poultry; when a resistant strain is present in an infected individual, medical treatments' effectiveness decreases resulting in the need for higher doses, and the increased likelihood of hospitalization and for longer periods of time (67).

Vegetables, water, poultry and pork products have been identified to be common sources of *Salmonella* contamination (13). Outside the United States, 33% of the foodborne outbreaks in Europe were associated with *S*. Enteritidis and 7% of the foods responsible for the contamination were pork and poultry meat and related products.

Hazard Characterization or dose response assessment

Dose response-characterization relates the probability of illness to the dose of the pathogen ingested (7). It is important to understand some characteristics of the pathogen, the host, and consider food matrix factors that will support the growth (18). To cause infection, *Salmonella* first needs to survive unfavorable conditions in the outside environment as well as inside the host such as the low pH in the stomach (18). In addition, *Salmonella* has to be able to attach itself to the intestinal epithelia and successfully compete with other flora naturally present in the GI track (18).These interactions and features of the microorganism could change depending on the serotype responsible of contamination. Host features such as age, gender, race, nutritional/social/economic status, condition of the immune system, and previous exposure could affect the outcome of exposure (28).

Exposure Assessment

Exposure assessment estimates the amount of *Salmonella* to which consumers are likely to be exposed (12). In order to apply probabilistic modeling it is of importance to have wide knowledge about the process to identify the stages that contribute the most to the final risk (19). When data is unavailable assumptions are made based on previous findings from scientific literature and from experts' opinion. Important inputs include, concentration of the pathogen in food, survival, growth or inhibition of the microorganism and patterns of consumption (28).

Risk characterization

Risk characterization integrates all three previous steps and estimates qualitative or quantitative the likelihood and impact of the adverse health on the evaluated population under the conditions stated in the hazard identification, hazard characterization and in the exposure assessment (28)

B. Monte Carlo simulation vs. Point estimates

Risk assessment frequently deals with uncertainty and variability. Variability comes from factors that have an impact in the outcome and overall risk but cannot be controlled because it is inherent to the heterogeneity on many factors related to the population. Examples include physiology, immune response, habits of food consumption among others. On the other hand, uncertainty can be reduced by data collection (73). Monte Carlo simulation technique generates a broader picture of the uncertainty by randomly picking values from a predefined probability distribution and uses those values for the mode (60).

II. Methods:

The first step was to represent the flow diagram of the processes to identify the sources where positive sample came from.

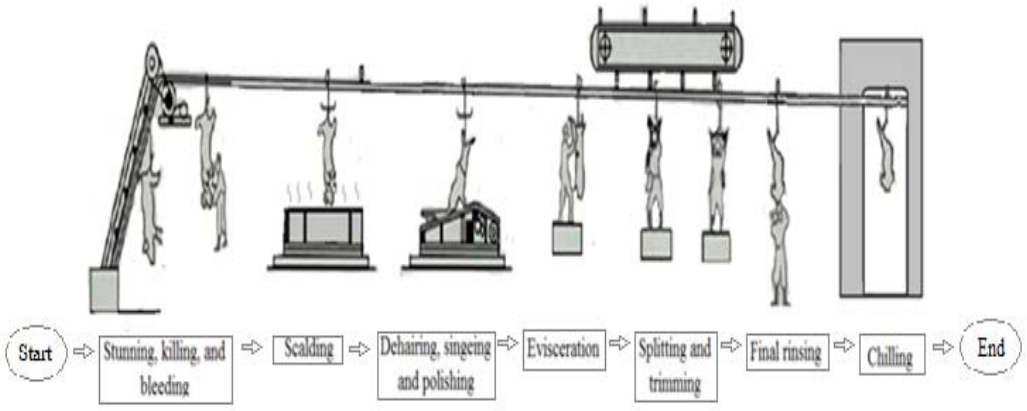
Table 20: *Salmonella* isolates collected form pork sources (plant D).

	P	O	IB	He	Hi	F	HP	E	T	GM
S. Anatum	2	1	1	4	1	3	1	1		
S. Derby									1	
S.Johannesburg		1								
S. Manhattan		1								
S. Mbandaka				1			1	1		
S. Saint Paul					1					
S. Typhimurium										2
Untypable	1									
Total Positives	**3**	**3**	**1**	**5**	**2**	**3**	**2**	**2**	**1**	**2**
Total Negatives	-	-	-	-	-	-	-	-	-	-

Key:
P= Pen feces, O= Offal, IB= Inedible barrel; H= Head; Hi= Hide; HP=Hide puller; F= Foot; E= Ears; T= Tongue, GM= Ground Meat.

Typically, a food safety objective should be defined and a sampling program should be planned to provide as much information as possible for the further development of risk assessment. It is very important to record the total number of samples collected from each source so the prevalence of the pathogen can be calculated to determine the source that contributes the most to the overall risk.

Figure 8: Flow diagram of pork slaughtering process



The Quantitative Microbial risk model was developed by Steve Stephens from the Food Processing Center at the University of Nebraska-Lincoln using Mathcad version 13.0. This model has been validated with data from other studies but validation with experimental data is still needed.

A. *Microbial Exposure and Dose Response Models*

Two equations need to be used to determine mathematically the dose and the risk of infection by consumption of a contaminated product.

Equation1:

$$\text{Dose} = (\text{Prevalence}) * (\text{Consumption}) * 10^{\text{Log Contamination}+\text{Log Growth}-\text{Log Reduction from cooking}}$$

Equation 2:

$$\text{Risk of Infection} = 1 - (1 + \frac{\text{Dose}}{\beta})^{-\alpha}$$

Where:

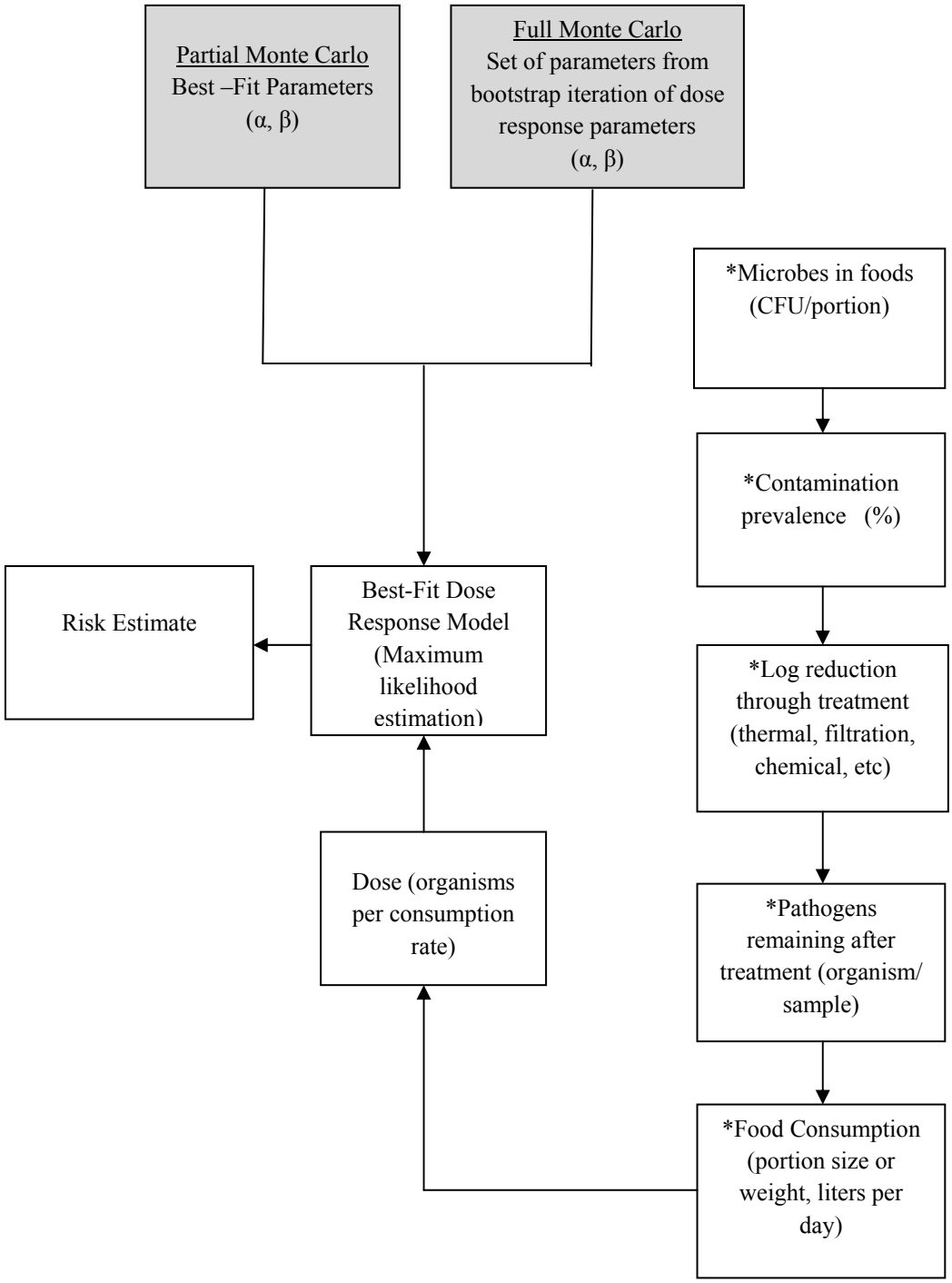
Dose= Number of pathogenic microorganisms ingested (log CFU), calculated in equation 1.

α,β= Dose response parameter, fitted using maximum likelihood estimation methods.

A. *Monte Carlo Simulation*

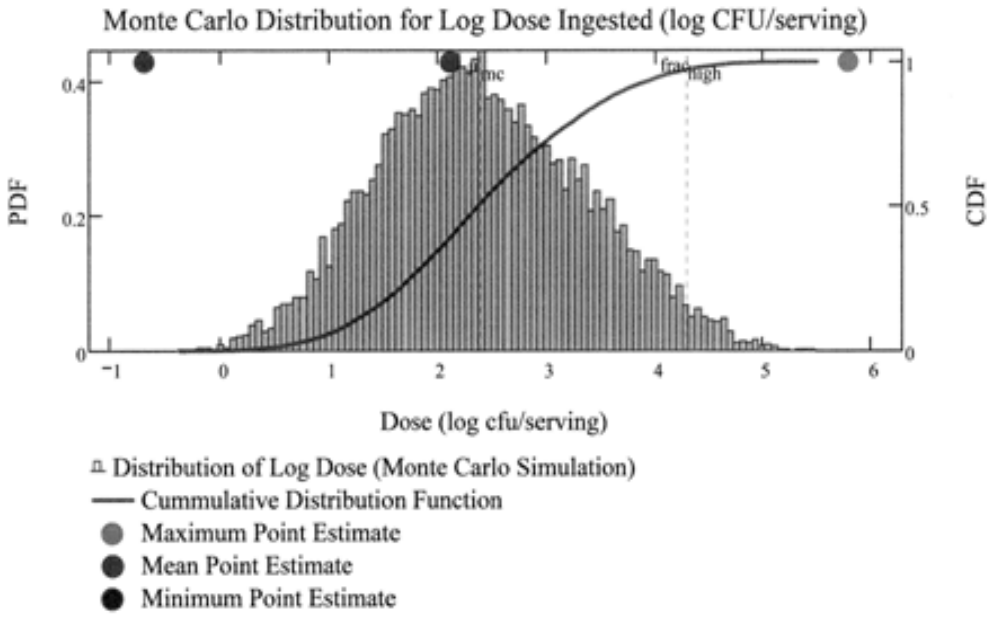
Figure 9 shows the inputs needed to perform a Monte Carlo simulation. Highlighted boxes refer to data than can be obtained from the literature. Inputs indicated in the boxes at the very right indicate data that can be obtained from laboratory experiments.

Figure 10: Monte Carlo Simulation Flowchart



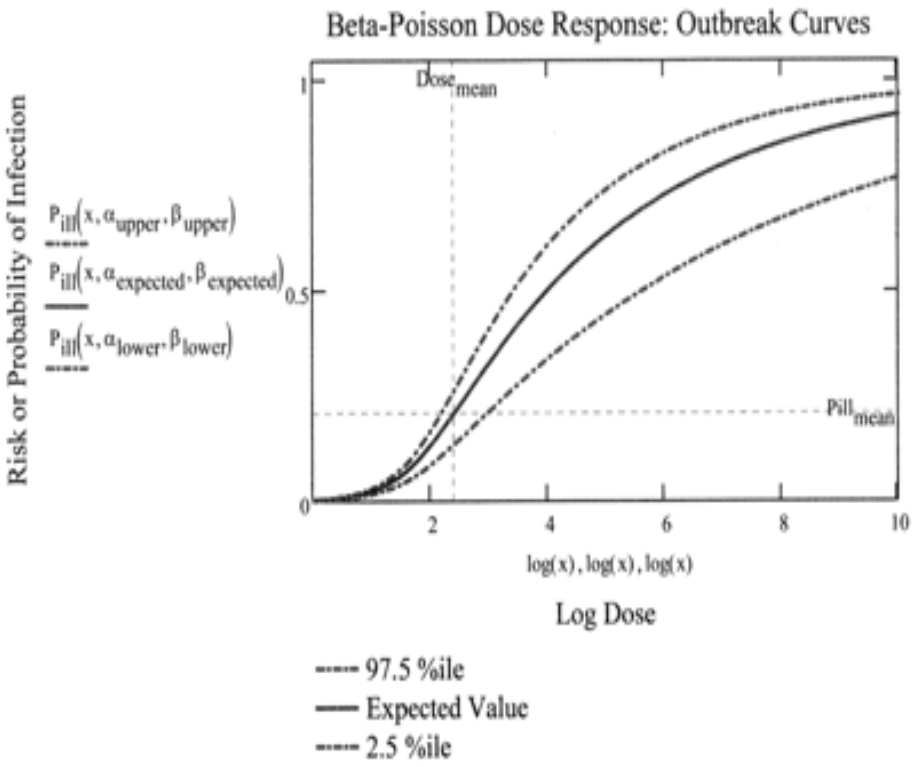
Distributions defining the food consumption, log reduction, concentration of microorganism in food and prevalence are generated using the inputs described above. All these distributions are finally combined to build a distribution for the dose ingested.

Figure 11: Monte Carlo simulation for bacteria Dose ingested



A separate Monte Carlo simulation is run to estimate the risk using the dose response model. The dose ingested is the input for the dose response model and the best fit parameters determined from the maximum likelihood estimation method. The simulation characterizes the uncertainty by finding the confidence interval after the dose response data is fitted to a dose response model.

Figure 12: Beta- Poisson Dose Response: Outbreak Curves



The dose response curve will indicate the log dose and the probability of illness by consuming a product contaminated with that log dose under the conditions established throughout the analysis and in the studied target population.

APENDIX A: Formulations of Solutions for Salmonella –PFGE Protocol

1 M Tris-HCl, pH 8.0
121.1 g Tris base
Dissolve in 650-700 ml type 1 water
Let come to room temperature
Final adjustments to pH
Dilute to 1000 ml with type 1 water
Sterilize by autoclaving

0.5 EDTA, pH 8.0
186.1 g Na_2 EDTA-2 H_2O
Add 800 ml type 1 water
Mix and adjust pH to 8 with about
50 ml 10 N NaOH

Phosphate Buffered saline (1X PBS)
0.8 g NaCl, 0.02 g KCl, 0.144 Na2HPO4
0.024 g KH2PO4 per liter
Adjust the pH to 7.4, autoclave

20 mg/ml Proteinase K stock solution
100 mg proteinase k powder (0.1 g)
5 ml sterile type 1 water
Mix and disperse into 500-600 µl
Volumes in 15 ml microcentrifuge tubes
Storage at -20 °C

10X Tris-Borate EDTA buffer (TBE)
0.9M Tris base (108 g)
0.9M Boric acid (55 g)
0.02M EDTA, pH 8.0 (40 ml 0.5m)
Dilute to 1000 ml with sterile type 1 water
Autoclave; discard if precipitate develops

10 % sarcosyl (N-laurilsarcosine sodium salt)
10 g sarcosyl
90 ml sterile type 1 water
Carefully add sarcosyl to water in sterile container
Dissolve by mixing gently and warm to 50-60 °C.
Wear a mask when weighing out and avoid aerosols.

Cell Lysis Buffer
50 mM Tris, 50 mM EDTA (pH 8.0)
1% sarcosine:
25 ml 1M Tris, pH 8.0
50 ml 0.5 M EDTA, pH 8.0
50 ml 10% sarcosyl
Dilute with 500 ml sterile type 1 water
Autoclave. At the time of use, add 0.1 mg/ml of proteinase K

10 N NaOH
400 g NaOH
Carefully dissolve in 800 ml sterile type 1 water. Cool to room temperature.
Dilute to 1000 ml with sterile Type 1 water.

Tris- EDTA Buffer (TE) pH 8.0
10 ml 1M Tris-HCl, pH 8.0
2 ml 0.5 m EDTA, pH 8.0

0.5X TBE Buffer
100 ml 10X TBE buffer
Dilute to 2000 ml with type 1 water

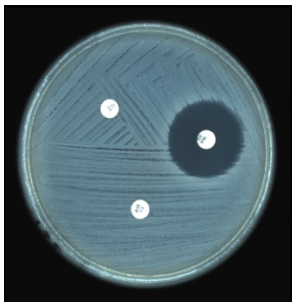
1% Seakem Gold Agarose (gels)
1 g agarose in 100 ml 0.5X TBE melt in microwave

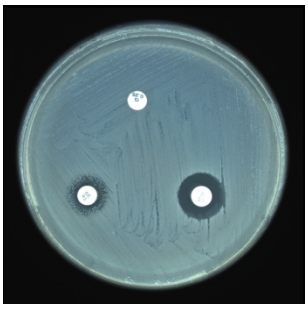
1% Seakem Gold Agarose (plugs)
1 g agarose in 100 ml 0.5 X TBE Melt in microwave

Adapted from CDC-PulseNet *Salmonella* Molecular Subtyping protocol

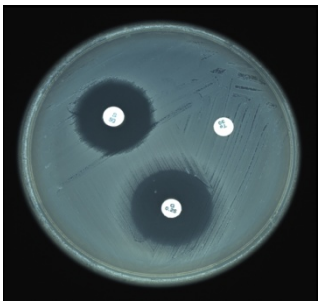
APENDIX B. Images from Antimicrobial resistant isolates

P2: *S*. Tyhimurium (Var. Copenhagen) , Pattern: G-AM- C

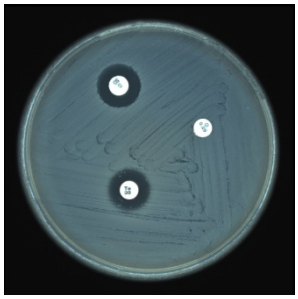


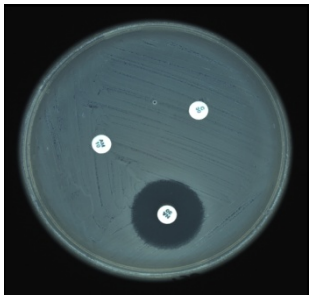


P3: *S*. Newport Pattern: Te

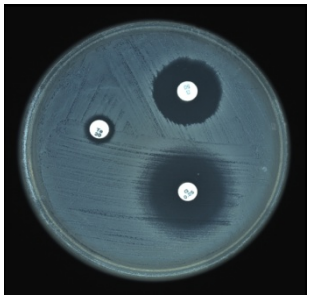


P6: *S*.Tyhimurium (Var. Copenhagen) , Pattern: G-AM-C

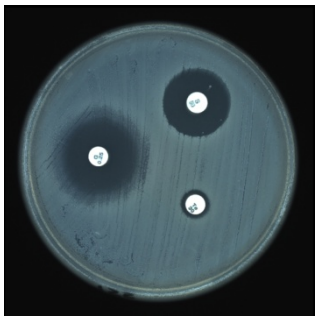




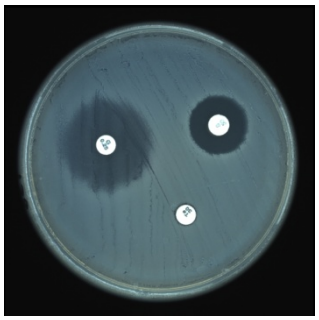
P27: *S.* Anatum Pattern: Te



P29 & P33 : *S.* Anatum Pattern: Te



P36: *S*. Mbandaka. Pattern: Te

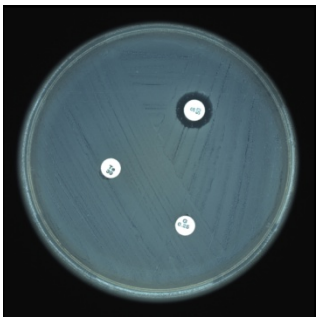


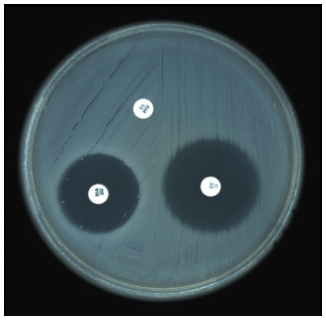
P38: *S.* Anatum Pattern: Te

P43: S. Johannesburg

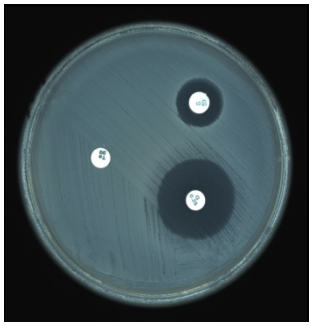
P46: *S.* Anatum Pattern: Te

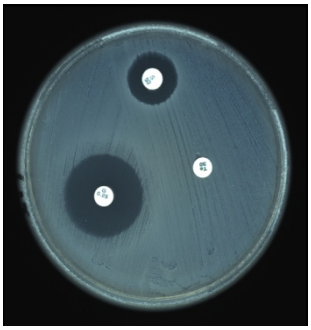
P52: S. Bovis- morbificans. Pattern: Te-G-AM

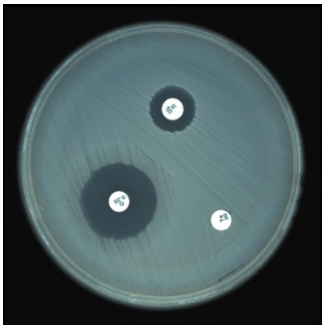




P49 ,P50 & P54 : S. Heildelberg. Pattern: Te

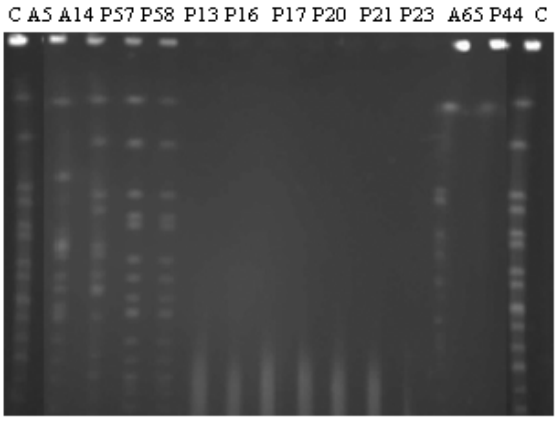






APENDIX C: PFGE images form fresh isolates from pork and poultry sources

091010



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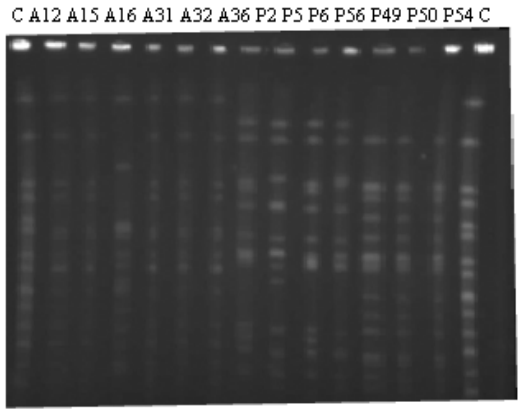

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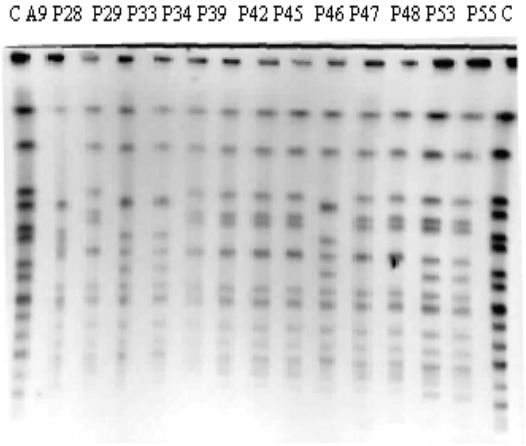

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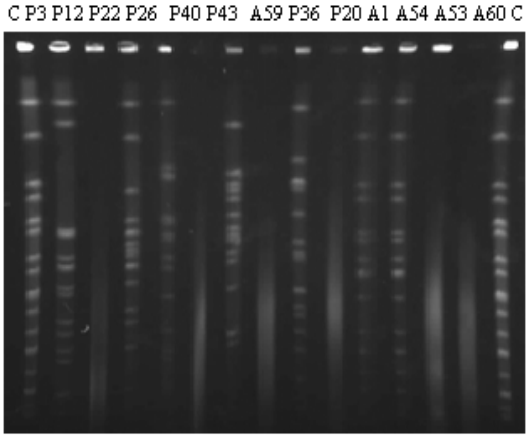

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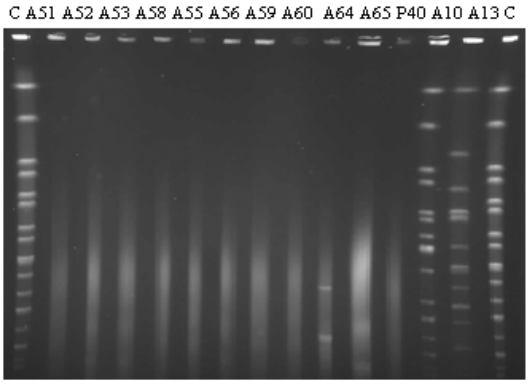

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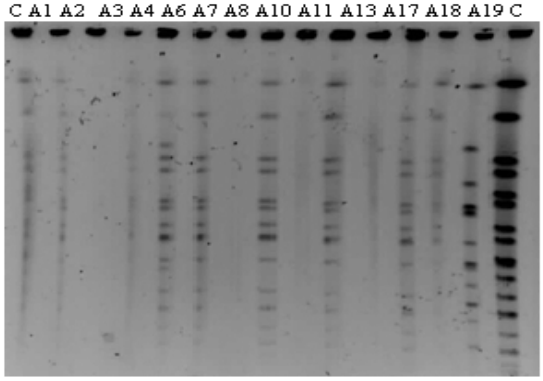

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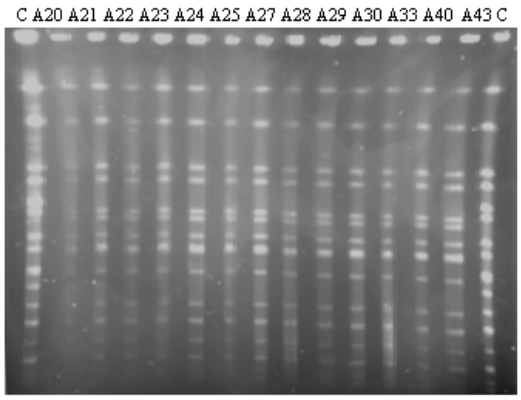

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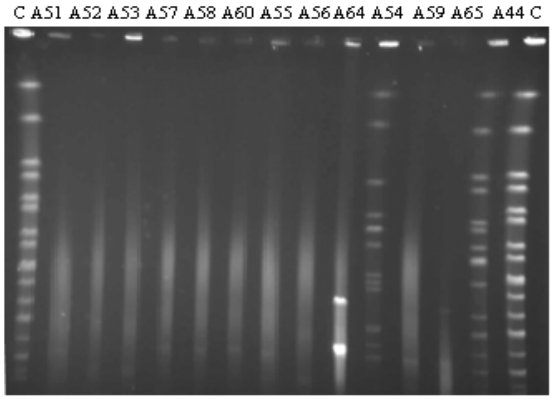

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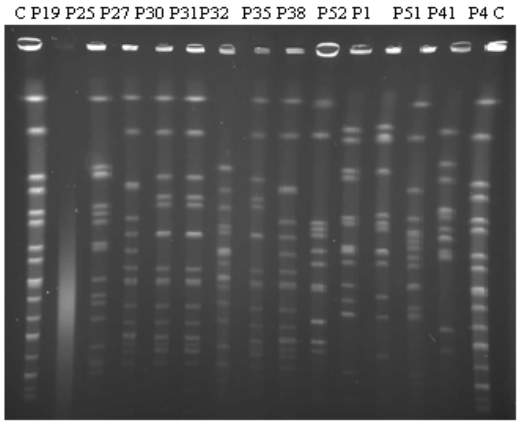


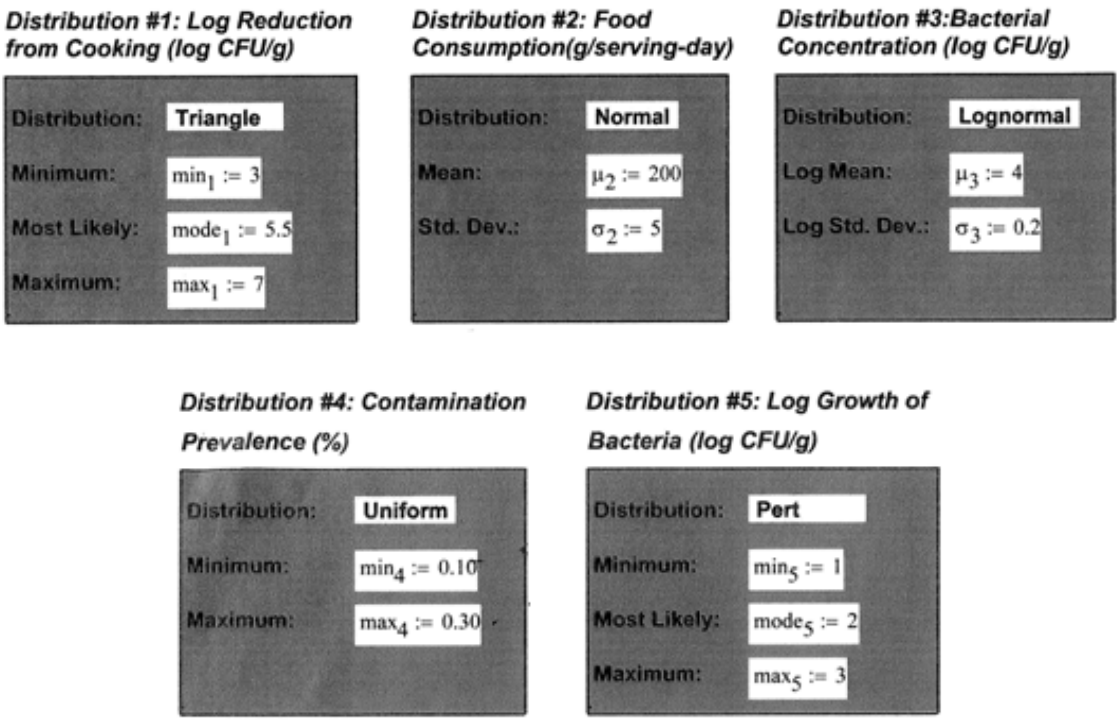
APENDIX D: Risk assessment inputs and outputs

Quantitative Microbial Risk Modeling:
Risk of Salmonellosis from Undercooked Pork Products
The Food Processing center
Steve Stephens and Yulie Meneses

1. Dose Ingested and Dose response Model Inputs

a) Define probability distributions and parameters for dose ingested model variables
Typically, parameter values are usually determined by fitting experimental data to a distribution. The distribution and parameters used in this experiment are hypothetical to illustrate the Monte Carlo simulation with an exposure model and do not represent actual experiment data.

Enter distribution parameters below for each random variable below.



b) Dose response data Inputs: Outbreak Data

The dose response parameters are fitted using the maximum likelihood estimation method. The experimental dose response data is needed (positive responses, negative responses and dose ingested). The uncertainty (confidence interval) in the model is

estimated using a bootstrapping routine involving a Monte Carlo simulation of dose response parameters.

Table 3.16. Beta-Poisson dose-response parameters that generate the approximate bounds shown in Figure 3.18.

	Alpha	Beta
Expected Value	0.1324	51.45
Lower Bound	0.0763	36.49
2.5th Percentile	0.0940	43.75
97.5th Percentile	0.1817	56.39
Upper Bound	0.2274	67.96

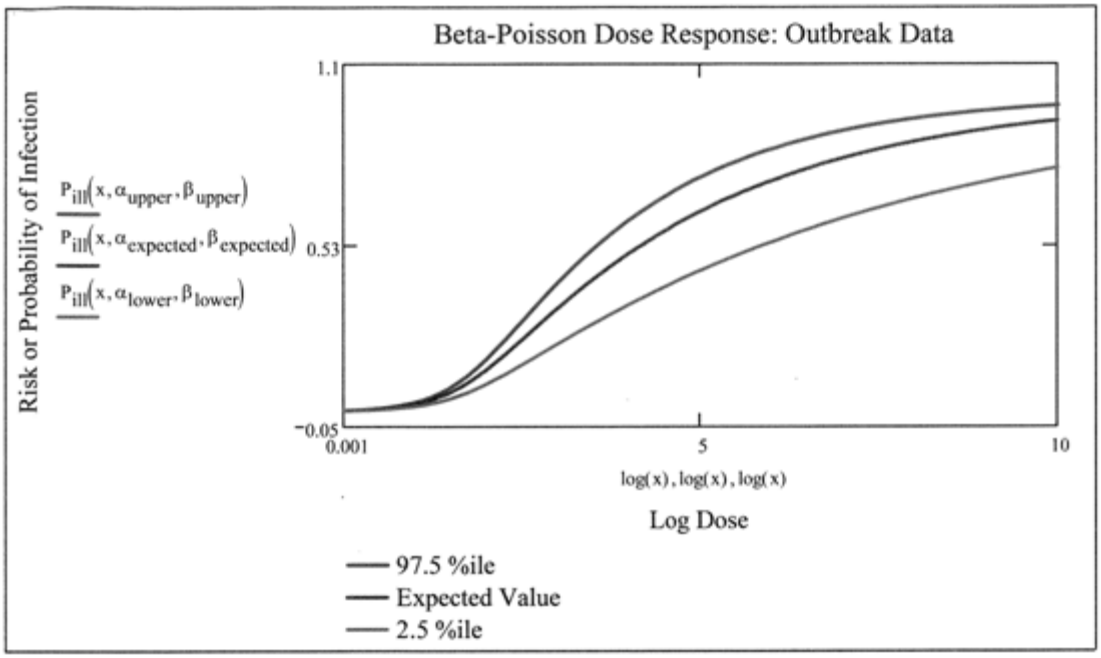
$\alpha_{upper} := 0.1817$ $\beta_{upper} := 56.39$

$\alpha_{expected} := 0.1324$ $\beta_{expected} := 51.45$

$\alpha_{lower} := 0.0763$ $\beta_{lower} := 43.75$

Beta-Poisson Dose Response Model: $P_{ill}(x,\alpha,\beta) := 1 - \left(1 + \frac{x}{\beta}\right)^{-\alpha}$

$x := \text{logspace}\left[0.001, (10)^{10}, 100\right]$



2.Deterministic and Probabilistic Dose Ingested Outputs

a. Deterministic (point estimate) Dose Ingested Calculations

The equation to calculate the deterministic/point estimates:
$\text{Dose} = (\text{Prevalence}) * (\text{Consumption}) * 10^{\text{Log Contamination} - \text{Log R}}$

Using the parameters above the point estimates values are as follows:

Dose

Minimum Value: $Dose_{min} := min_4 \cdot \mu_2 \cdot 10^{\mu_3 + min_5 - max_1}$ $Dose_{min} = 0.2$

Mean Value: $Dose_{mean} := \left(\frac{max_4 + min_4}{2}\right) \cdot \mu_2 \cdot 10^{\mu_3 + mode_5 - mode_1}$ $Dose_{mean} = 126.5$

Maximum Value: $Dose_{max} := max_4 \cdot \mu_2 \cdot 10^{\mu_3 + max_5 - min_1}$ $Dose_{max} = 600000$

Log Dose

Minimum Value: $LogDose_{min} := \log(Dose_{min})$ $LogDose_{min} = -0.699$

Mean Value: $LogDose_{mean} := \log(Dose_{mean})$ $LogDose_{mean} = 2.102$

Maximum Value: $LogDose_{max} := \log(Dose_{max})$ $LogDose_{max} = 5.778$

b. Probabilistic- Monte Carlo Simulation for Dose Ingested Random Variables
Enter number of Monte Carlo simulations and desired confidence intervals to observe for the dose ingested and dose response models.

Dose Ingested Simulation

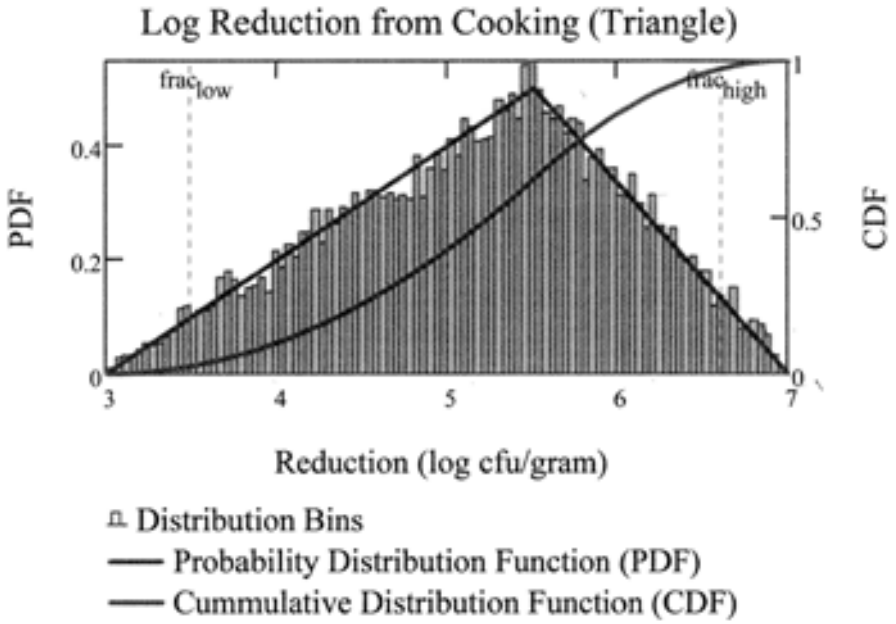
Number of Simulations: npts := 10000

Number of Distribution Bins: nbins := 100

Confidence Interval: CI := 95%

c. Probability Distribution outputs for exposure variables

Triangle Distribution: Log Reduction from Cooking (log cfu/ gram)

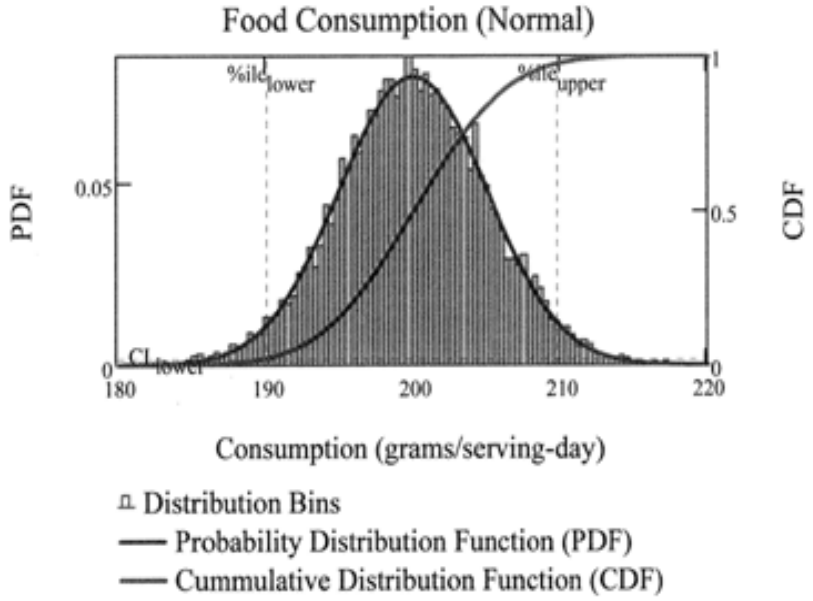


Triangle Distribution Parameter Values for Log Reduction

Parameter	Theoretical	Monte Carlo
Mean	$\mu_t = 5.167$	$\mu_{mc} = 5.178$
Std. dev.	$\sigma_t = 0.825$	$\sigma_{mc} = 0.836$
Minimum	$min_t = 3$	$min_{mc} = 3.022$
Most Likely	$mode_t = 5.5$	
Maximum	$max_t = 7$	$max_{mc} = 6.975$

Confidence Interval:	CI = 95.%		
Confidence Limits		**Percentile Value**	**Fractile Value**
	Lower	$CI_{lower} = 0.025$	$frac_{low} = 3.5$
	Upper	$CI_{upper} = 0.975$	$frac_{high} = 6.613$

Normal Distribution: Food Consumption (gram/ serving-day)



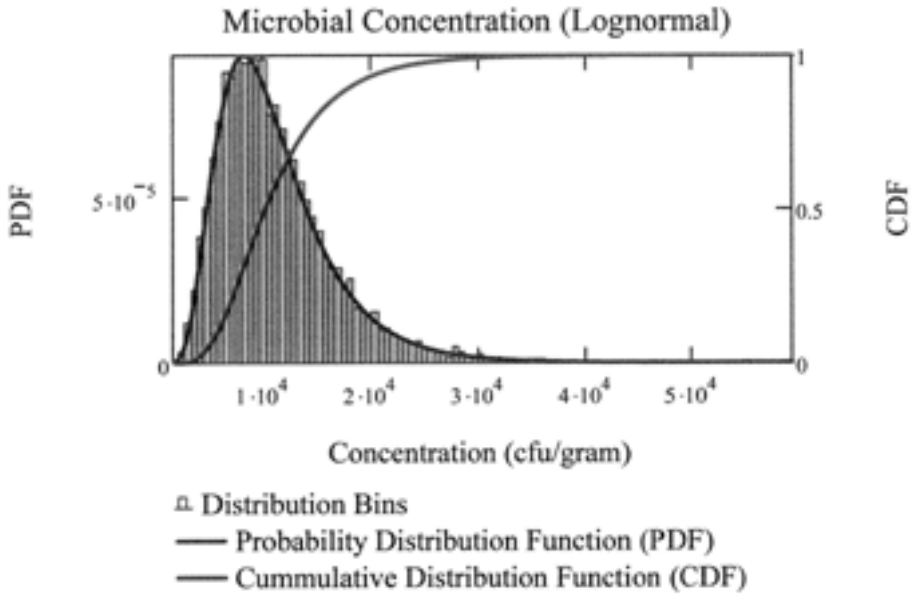
Normal Distribution Parameter Values for Consumption

Parameter	Theoretical	Monte Carlo
Mean	$\mu_2 = 200$	$\mu_{mc} = 199.979$
Std. dev.	$\sigma_2 = 5$	$\sigma_{mc} = 5.017$
Minimum	∞	$min_{mc} = 180.573$
Maximum	∞	$max_{mc} = 217.729$

Confidence Interval: CI = 95.%

Confidence Limits	Percentile Value	Fractile Value
Lower	$CL_{lower} = 2.5\%$	$frac_{low} = 190.025$
Upper	$CL_{upper} = 97.5\%$	$frac_{high} = 209.78$

Lognormal Distribution: Bacterial Concentration (log_cfu/gram) on Raw Product



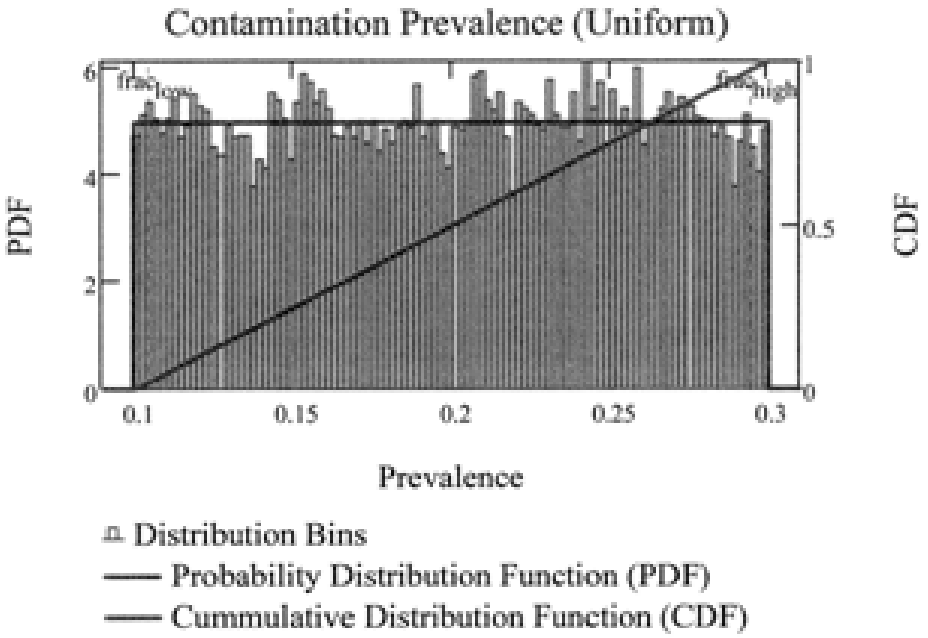
Lognormal Distribution Parameter Values for Conc.

Parameter	Theoretical	Monte Carlo
Log Mean	$\mu_3 = 4$	$\log(\mu_{mc}) = 4.047$
Log Std. dev.	$\sigma_3 = 0.2$	$\log(\sigma_{mc}) = 3.733$
Minimum	0	$\log(min_{mc}) = 3.208$
Maximum	∞	$\log(max_{mc}) = 4.772$

Confidence Interval: $CI = 95\%$

Confidence Limits	Percentile Value	Fractile Value
Lower	$CL_{lower} = 2.5\%$	$\log(frac_{low}) = 3.608$
Upper	$CL_{upper} = 97.5\%$	$\log(frac_{high}) = 4.392$

Uniform Distribution: Contamination Prevalence (%)



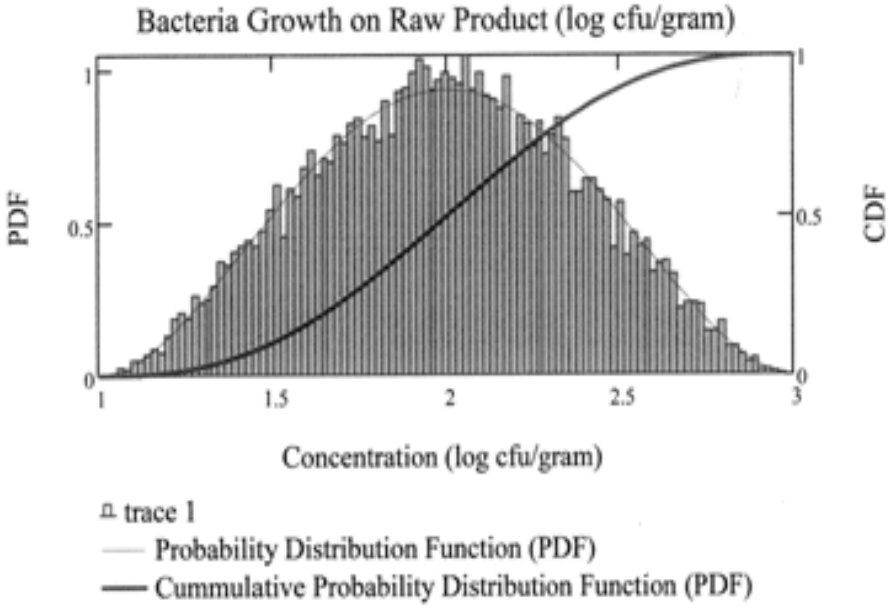
Uniform Distribution Parameter Values for Prevalence

Parameter	Theoretical	Monte Carlo
Mean	$\mu_q = 0.2$	$\mu_{mc} = 0.2$
Std. dev.	$\sigma_q = 0.058$	$\sigma_{mc} = 0.057$
Minimum	$min_q = 0.1$	$min_{mc} = 0.1$
Maximum	$max_q = 0.3$	$max_{mc} = 0.3$

Confidence Interval: CI = 95.0%

Confidence Limits	Percentile Value	Fractile Value
Lower	$CL_{lower} = 2.5\%$	$frac_{low} = 0.105$
Upper	$CL_{upper} = 97.5\%$	$frac_{high} = 0.295$

Pert Distribution: Bacteria Growth on Raw Product (log cfu/gram)



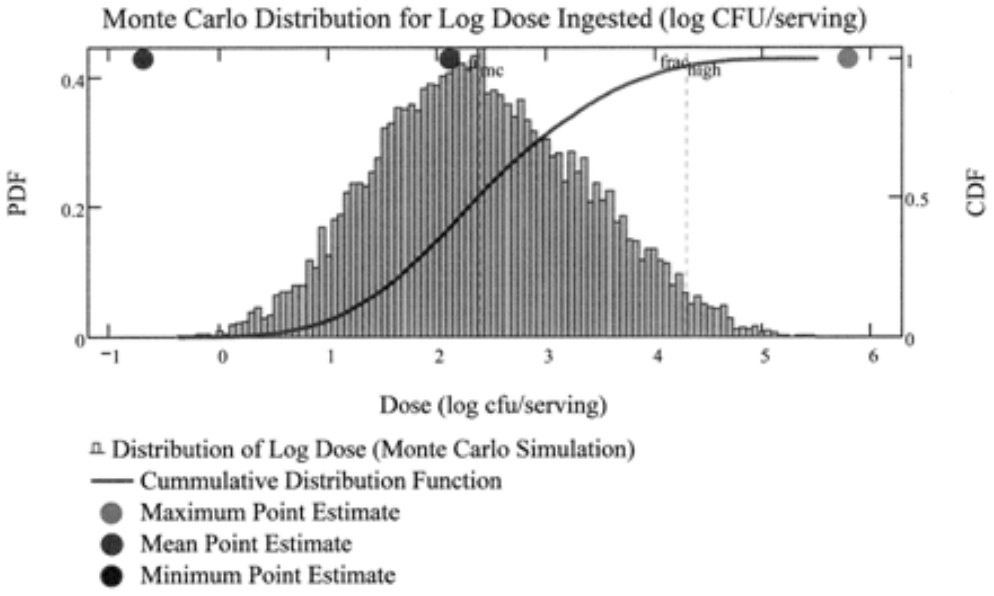
Beta Distribution Parameter Values for Prevalence

Parameter	Theoretical	Monte Carlo
Mean	$\mu = 2$	$\text{mean}(Xmc_{pert}) = 1.997$
Std. dev.		
Minimum	$min_S = 1$	$min_{mc} = 0.1$
Maximum	$max_S = 3$	$max_{mc} = 0.3$

Confidence Interval: CI = 95 %

Confidence Limits	Percentile Value	Fractile Value
Lower		
Upper		

Monte Carlo Simulation for Bacteria Dose Ingested



Deterministic and Probabilistic Values for Log Dose

Parameter	Point Estimate	Monte Carlo
Log Mean	$LogDose_{mean} = 2.102$	$\mu_{mc} = 2.403$
Log Std. dev.		$\sigma_{mc} = 0.943$
Minimum	$LogDose_{min} = -0.699$	$min_{mc} = -0.38$
Maximum	$LogDose_{max} = 5.778$	$max_{mc} = 5.484$

Confidence Interval: CI = 95 %

Confidence Limits	Percentile	Fractile Value
Lower	$CL_{lower} = 2.5$ %	$frac_{low} = 0.669$
Upper	$CL_{upper} = 97.5$ %	$frac_{high} = 4.294$

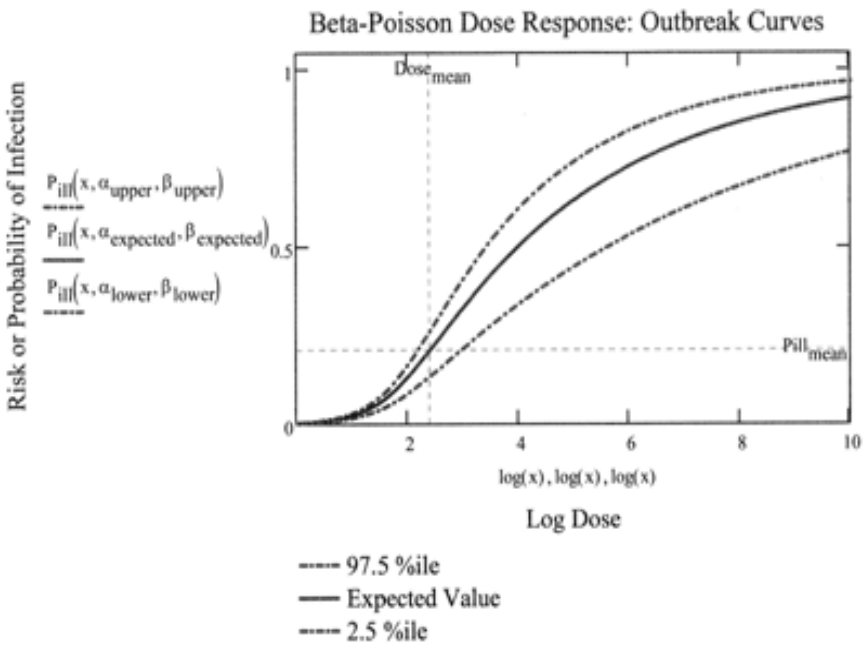
Risk Outputs –Dose Response and Confidence Region

Beta-Poission Dose Response: $P_{ill}(x,\alpha,\beta) := 1 - \left(1 + \frac{x}{\beta}\right)^{-\alpha}$

Range varialbe for plotting dose response curves: $x := \text{logspace}\left[0.0001, (10)^{10}, 100\right]$

Mean Dose: $Dose_{mean} := \log\left(10^{\mu_{mc}}\right)$ $Dose_{mean} = 2.403$

$$Pill_{mean} := P_{ill}\left(10^{\mu_{mc}}, \alpha_{expected}, \beta_{expected}\right)$$



Risk of Infection Estimates:

2.5 %ile Risk: $P_{ill}\left(10^{Dose_{mean}}, \alpha_{lower}, \beta_{lower}\right) = 0.136$

Mean Risk: $P_{ill}\left(10^{Dose_{mean}}, \alpha_{expected}, \beta_{expected}\right) = 0.21$

97.5 %ile Risk: $P_{ill}\left(10^{Dose_{mean}}, \alpha_{upper}, \beta_{upper}\right) = 0.266$

If an individual ingests a mean log dose ($Dose_{mean} = 2.403$) the risk of that individual becoming infected or ill is ($Pill_{mean} = 0.21$).

Uppper Dose (97.5 %ile) Risk Estimates:

2.5 %ile Risk: $P_{ill}\left(10^{frac_{high}}, \alpha_{lower}, \beta_{lower}\right) = 0.373$

Mean Risk: $P_{ill}\left(10^{frac_{high}}, \alpha_{expected}, \beta_{expected}\right) = 0.545$

97.5 %ile Risk: $P_{ill}\left(10^{frac_{high}}, \alpha_{upper}, \beta_{upper}\right) = 0.655$

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CHAPTER VII

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